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(54) **CYCLOISOMALTOTETRAOSE, CYCLOISOMALTOTETRAOSE PRODUCTION ENZYME, AND PRODUCTION METHODS AND USES THEREOF**

(57) The present invention has objects to provide a novel cyclic saccharide composed of D-glucose as constituents, a novel enzyme forming the cyclic saccharide, processes for producing the same, and uses thereof. The present invention solves the above objects by providing cycloisomaltotetraose having a structure in which four D-glucose molecules are bound in cyclic form via α -1,6 glucosidic linkages, a cycloisomaltotetraose-forming enzyme, a DNA encoding the enzyme, processes for producing the same, and uses thereof.

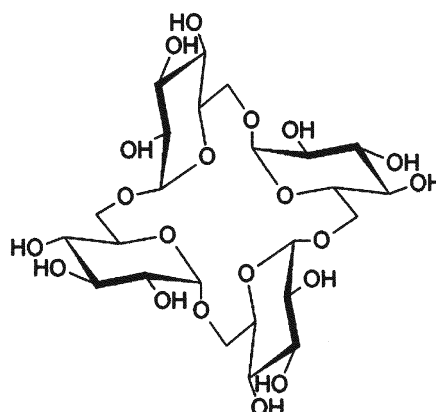


FIG.8

DescriptionTechnical Field

5 **[0001]** The present invention relates to a novel cyclic saccharide composed of D-glucose as constituents, a novel enzyme which forms the cyclic saccharide, their preparation and uses.

Background Art

10 **[0002]** In the field of saccharides, various cyclic saccharides, produced by enzymatic reactions using α -1,4 glucan composed by D-glucose such as starch or partial starch hydrolysates as material, are known. Non-Patent Literature 1 discloses α - β - and γ -cyclodextrins, saccharides having cyclic structures that 6 to 8 D-glucose molecules are bound in cyclic form *via* α -1,4 glucosidic linkages, obtainable by allowing "macerans amylase" to act on starch. Currently, these cyclodextrins are produced on an industrial scale and utilized in various applications using their characteristics, such as non-reducing power, tastelessness, and inclusion ability. While, Patent Literature 1 discloses a cyclic tetrasaccharide, having a structure of binding four glucose molecules *via* alternating α -1,3 and α -1,6 glucosidic linkages, i.e., *cyclo*{ $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -D-glucopyranosyl-(1 $\rightarrow$)}, which is obtainable by allowing α -isomaltosylglucosaccharide-forming enzyme and α -isomaltosyl-transferring enzyme to act on starch or its partial hydrolysate. Further, Patent Literature 2 discloses a cyclic tetrasaccharide, having a structure of binding four glucose molecules *via* alternating α -1,4 and α -1,6 glucosidic linkages, i.e., *cyclo*{ $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 4)- α -D-glucopyranosyl-(1 $\rightarrow$)} (also known as "cyclic maltosylmaltose"), which is obtainable by allowing a cyclic maltosylmaltose-forming enzyme to act on starch or its partial hydrolysates. Furthermore, Patent Literature 3 discloses a cyclic pentasaccharide having a structure of binding C-1 position of the reducing end glucose of maltopentaose to the hydroxy group at the C-6 position of the non-reducing end glucose in the same maltopentaose molecule *via* α -1,6 glucosidic linkage to form cyclic structure ("isocyclomaltopentaose") and a cyclic hexasaccharide having a structure of binding C-1 position of the reducing end glucose of maltohexaose to the hydroxy group at the C-6 position of the non-reducing end glucose in the same maltohexaose molecule *via* α -1,6 glucosidic linkage to form cyclic structure ("isocyclomaltohexaose"). These cyclic saccharides have an inclusion ability because they have a cyclic structure, and exhibit the effect of stabilizing the volatile organic substances. Further, since they are non-reducing saccharides, they can be used for processing without causing an aminocarbonyl reaction, browning, and degradation. Therefore, they are expected to be utilized in various fields by taking advantage of each characteristic.

25 **[0003]** On the other hand, as another α -glucan composed by D-glucose, dextran, an α -1,6 glucan having a basic structure in which D-glucose molecules are bound in a chain *via* α -1,6 glucosidic linkages, has been known. In addition, cycloisomaltooligosaccharides (also called as "cyclic isomaltooligosaccharide" or "cyclodextran") have been reported as cyclic saccharides obtainable by an enzymatic reaction using dextran as a material. Patent Literature 4 and Non-Patent Literature 2 disclose cycloisomaltoheptaose (CI-7), cycloisomaltooctaose (CI-8) and cycloisomaltononaose (CI-9), cyclic saccharides in which 7 to 9 D-glucose molecules were bound *via* α -1,6 glucosidic linkages, obtainable by allowing an enzyme derived from a microorganism of the genus *Bacillus*. Further, Patent Literature 5 and Non-Patent Literature 3 disclose CI-10 to CI-16, having higher glucose polymerization degrees of 10 to 16, and Patent Literature 6 discloses CI-13 to CI-33, having more higher glucose polymerization degrees of 13 to 33. However, cycloisomaltooligosaccharides having glucose polymerization degrees of less than 7 have not been known at all.

30 **[0004]** If other cyclic saccharide having D-glucose molecule as a component, distinct from the above saccharides, would be provided, application thereof for various uses can be expected.

Prior Art LiteraturePatent Literature

45 **[0005]**

Patent Literature 1: International Patent Application WO2001/90338A1 Pamphlet
 Patent Literature 2: International Patent Application WO2005/21564A1 Pamphlet
 Patent Literature 3: International Patent Application WO2006/35725A1 Pamphlet
 55 Patent Literature 4: Japanese Patent Kokai No. H06-0197783
 Patent Literature 5: Japanese Patent Kokai No. 2004-166624
 Patent Literature 6: Japanese Patent Kokai No. 2005-192510

Non-Patent Literature**[0006]**

Non-Patent Literature 1: Journal of American Chemical Society, USA, (1949), Vol. 71, pp. 353-358
 Non-Patent Literature 2: Biosci. Biotech. Biochem., Vol. 57, pp. 1225-1227 (1993)
 Non-Patent Literature 3: Biosci. Biotech. Biochem., Vol. 72, pp. 3277-3280 (2008)

DISCLOSURE OF INVENTIONObject of the Invention

[0007] Objects of the present invention are to provide a novel cyclic saccharide composed of D-glucose as constituents, a novel enzyme forming the cyclic saccharide, processes for producing the same, and uses thereof.

Mean to Attain the Object

[0008] To solve the above objects, the present inventors have extensively screened microorganisms capable of producing a novel enzyme which forms a novel cyclic saccharide when allowed to act on dextran or its partial hydrolysate, having α -1,6 glucan, a glucose polymer which is formed by binding D-glucose molecules in a linear fashion *via* α -1,6 glucosidic linkages, as a basic structure.

[0009] As the results, the present inventors found that a strain D1110 belonging to the genus *Agreia* and a strain D2006 belonging to the genus *Microbacterium*, which are newly isolated from soil samples, produce novel enzymes, and also found that a remarkable amount of a novel saccharide having a structure of binding four D-glucose molecules to form cyclic structure *via* α -1,6 glucosidic linkages (hereinafter, simply abbreviated as " α -1,6 linkage"), can be produced by the action of the novel enzyme from α -1,6 glucan, or dextran and its partial hydrolysate, having the α -1,6 glucan as the basic structure.

[0010] The present inventors have cultured the microorganisms having the ability of producing the novel enzyme which forms the cycloisomaltotetraose, purified the cycloisomaltotetraose-forming enzyme from the culture and revealed its properties, and then established a process for producing the same. Further, the present inventors also established a DNA encoding the enzyme, a recombinant DNA containing the DNA, and transformant comprising the recombinant DNA. Furthermore, the present inventors also established a process for producing cycloisomaltotetraose or a saccharide composition containing the same by using the enzyme and uses thereof, and then completed the present invention.

[0011] The present invention solves the above objects by providing a novel saccharide, cycloisomaltotetraose having a structure of which four D-glucose molecules are bound in cyclic form *via* α -1,6 glucosidic linkages, a novel cycloisomaltotetraose-forming enzyme which forms the saccharide, a DNA encoding the enzyme, a recombinant DNA comprising the DNA and a transformant having the recombinant DNA, processes for producing those, a composition comprising cycloisomaltotetraose or a saccharide composition comprising the same such as foods and beverages, luxury grocery items, feeds, baits, cosmetics, pharmaceuticals, industrial supplies, etc.

Effect of the Invention

[0012] According to the present invention, cycloisomaltotetraose, a novel cyclic saccharide which has not been ever known, and a saccharide composition comprising the same can be provided, and the present invention enables the use of those in various fields including foods and beverages, cosmetics, and pharmaceuticals.

BRIEF DESCRIPTION OF DRAWINGS**[0013]**

FIG. 1 shows a TLC chromatogram of the enzyme reaction mixture obtained by allowing a crude enzyme from a microorganism strain D1110 to act on dextran as a substrate.

FIG. 2 shows a HPLC chromatogram of the purified preparation of "Unknown Saccharide I".

FIG. 3 shows a ^1H -NMR spectrum of the purified preparation of "Unknown Saccharide I".

FIG. 4 shows a ^1H - ^1H COSY spectrum of the purified preparation of "Unknown Saccharide I".

FIG. 5 shows a ^{13}C -NMR spectrum of the purified preparation of "Unknown Saccharide I".

FIG. 6 shows a HSQC spectrum of the purified preparation of "Unknown Saccharide I".

FIG. 7 shows a HMBC spectrum of the purified preparation of "Unknown Saccharide I".

FIG. 8 shows the structure of cycloisomaltotetraose of the present invention.

FIG.9 shows a micrograph of crystals of cycloisomaltotetraose (Magnification: x 400).

FIG. 10 shows a powder X-ray diffraction pattern of crystalline cycloisomaltotetraose pentahydrate.

FIG. 11 shows the optimum temperature of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110.

FIG. 12 shows the optimum pH of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110.

FIG. 13 shows the thermal stability of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110.

FIG. 14 shows the pH stability of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110.

FIG. 15 shows the optimum temperature of cycloisomaltotetraose-forming enzyme derived from *Microbacterium trichothecenolyticum* D2006.

FIG. 16 shows the optimum pH of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006.

FIG. 17 shows the thermal stability of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006.

FIG. 18 shows the pH stability of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006.

FIG. 19 shows the homology between amino acid sequences encoded by ORF9038 and ORF5328, genes of cycloisomaltotetraose-forming enzymes from *Agreia* sp. D1110 and *Microbacterium trichothecenolyticum* D2006, respectively.

FIG. 20 shows a recombinant DNA, pRSET A-ORF9038. In the figure, the black bold line means a DNA encoding cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110.

FIG. 21 shows a HPLC chromatogram of a reaction mixture obtained by allowing a supernatant of cell extract prepared by culturing a recombinant *E. coli* E9038 having the recombinant DNA, pRSET A-ORF9038 to act on dextran as a substrate.

FIG. 22 shows a HPLC chromatogram of a saccharide composition containing glucosyl-derivatives of cycloisomaltotetraose, obtained by the steps of allowing cyclomaltodextrin glucanotransferase to act on a glucosyl donor, α -cyclodextrin in a presence of an acceptor, cycloisomaltotetraose for glycosyl transferring reaction, hydrolyzing the resulting products by glucoamylase, and subjecting the resulting reaction mixture to alkaline treatment.

FIG. 23 shows the structures of two glucosyl-derivatives of cycloisomaltotetraose.

MODE FOR CARRYING OUT THE INVENTION

[0014] The present invention relates to a novel cyclic saccharide, cycloisomaltotetraose. Cycloisomaltotetraose as referred to in the present invention means a cyclic saccharide having a structure in which four D-glucose molecules are bound in cyclic form via α -1,6 glucosidic linkages, i.e., $cyclo\{\rightarrow 6\}$ - α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$ 6)- α -D-glucopyranosyl-(1 $\rightarrow$) (See FIG. 8).

[0015] Cycloisomaltotetraose of the present invention is a novel and ever unknown saccharide, firstly found by the present inventors in a reaction mixture obtained by allowing a culture broth of a microorganism isolated from a soil as a crude enzyme to act on a substrate, dextran. The present invention encompasses cycloisomaltotetraose independently by its source and process for producing it, as far as it has the above structure.

[0016] Cycloisomaltotetraose of the present invention encompasses that in a crystalline form as another embodiment. The crystal of cycloisomaltotetraose of the present invention is, usually, in the form of pentahydrate crystal.

[0017] The crystalline cycloisomaltotetraose pentahydrate of the present invention shows characteristic diffraction peaks at the diffraction angles (2θ) of 9.0°, 9.8°, 11.8°, 13.1°, and 19.1° in its powder X-ray diffraction pattern.

[0018] The present invention includes, as another embodiment, a saccharide composition comprising cycloisomaltotetraose which comprises cycloisomaltotetraose and/or crystalline cycloisomaltotetraose together with other saccharides. The form of the saccharide composition comprising cycloisomaltotetraose may be any of a syrup, powder, powder containing crystals or solid.

[0019] The present invention includes, as another embodiment, a glucosyl derivative of cycloisomaltotetraose having a structure in which one or two D-glucose molecules are bound to cycloisomaltotetraose. The binding mode of D-glucose to cycloisomaltotetraose in the glucosyl derivative is not particularly restricted, and may be any of α -1,2, α -1,3, or α -1,4 linkage. A glucosyl derivatives in which one D-glucose molecule is bound to one glucose residue of the four glucose residues constituting the cycloisomaltotetraose via α -1,3 linkage is present in trace amounts in the reaction mixture obtained by cycloisomaltotetraose-forming reaction of the enzyme described later. Further, as illustrated in the examples below, glucosyl derivatives of cycloisomaltotetraose can be produced and collected by the steps of allowing an appropriate glycosyltransferase to act on an appropriate glycosyl donor in the presence of cycloisomaltotetraose as an acceptor and enzymatically hydrolyzing the resulting transglycosylation product. As the glycosyltransferase, for example, α -glucosidase, β -glucosidase, α -amylase, cyclomaltodextrin glucanotransferase (CGTase), etc. can be used. For the enzymatic hydrolysis of the transglycosylation product, for example, glucoamylase, β -amylase, etc. can be used. Furthermore, by

selecting the glycosyl donor and the glycosyltransferase, glycosyl derivatives of cycloisomaltotetraose in which a saccharide other than D-glucose is transferred to cycloisomaltotetraose, for example, fructosyl derivatives, galactosyl derivatives, etc. can also be advantageously produced and collected.

[0020] Cycloisomaltotetraose-forming enzyme as referred to in the present invention means any enzyme which acts on α -1,6 glucan with a glucose polymerization degree of 4 or higher, dextran, or partial dextran hydrolysate and catalyzes the reaction of forming cycloisomaltotetraose. Cycloisomaltotetraose-forming enzyme of the present invention encompasses any enzyme catalyzing the above reaction without being restricted by its source, form, and purity, and by the reaction mechanism of acting to α -1,6 glucan having a glucose polymerization degree of 4 or higher, dextran or partial dextran hydrolysate and forming cycloisomaltotetraose. For example, when cycloisomaltotetraose-forming enzyme of the present invention is allowed to act on dextran, it is presumed that the enzyme cleaves α -1,6 linkages of dextran in isomaltotetraose units, and catalyzes an intramolecular α -1,6 transglycosylation of transferring the C1 position of the reducing end glucose of the isomaltotetraose to the hydroxyl group at the C6 position of the non-reducing end glucose of the same molecule to form cycloisomaltotetraose by cyclization. In a preferred example, cycloisomaltotetraose-forming enzyme of the present invention also has an activity of further acting on the once formed cycloisomaltotetraose and hydrolyzing to cleave the cyclic structure to form isomaltotetraose.

[0021] The enzyme activity of cycloisomaltotetraose-forming enzyme of the present invention can be assayed as follows:

A substrate solution is prepared by dissolving dextran or partial dextran hydrolysate in 20 mM acetate buffer (pH 6.0) to give a concentration of 1.11% (w/v). To 0.9 mL of the substrate solution, 0.1 mL of an enzyme solution, which prepared by 20 mM acetate buffer solution (pH 6.0) is added, and the mixture solution is incubated at 40°C to start the reaction. Then, 0.4 mL each of the reaction mixture is sampled at reaction times of 0.5 min and 30.5 min, and the reaction is stopped by heating at 100°C for 10 min for inactivate the enzyme. Successively, the amount of cycloisomaltotetraose in each reaction mixture is determined by HPLC described later in Experiment 1, and then the amount of cycloisomaltotetraose formed in 30 min-reaction is calculated by subtracting the amount of cycloisomaltotetraose at 0.5 min-reaction from that at 30.5 min-reaction. One unit activity (U) of cycloisomaltotetraose-forming enzyme is defined as the amount of enzyme which forms one μ mole of cycloisomaltotetraose per minute under the above conditions.

[0022] As a concrete example of cycloisomaltotetraose-forming enzyme of the present invention, the enzyme having the following physicochemical properties can be listed.

- (1) Molecular weight
86,000 $\pm$ 10,000 daltons when determined on SDS-polyacrylamide gel electrophoresis;
- (2) Optimum temperature
35 to 40°C when reacted at pH 6.0 for 30 min;
- (3) Optimum pH
pH 5.5 to 6.0 when reacted at 40°C for 30 min;
- (4) Thermal stability
Stable up to 40°C when incubated at pH 6.0 for 30 min; and
- (5) pH Stability
Stable in a pH range of 5.0 to 10.0 when incubated at 4°C for 16 hours;

[0023] As another concrete example of cycloisomaltotetraose-forming enzyme of the present invention, the enzyme having the following physicochemical properties can be listed.

- (1) Molecular weight
86,000 $\pm$ 10,000 daltons when determined on SDS-polyacrylamide gel electrophoresis;
- (2) Optimum temperature
40°C when reacted at pH 6.0 for 30 min;
- (3) Optimum pH
About pH 5.5 when reacted at 40°C for 30 min;
- (4) Thermal stability
Stable up to 35°C when incubated at pH 6.0 for 30 min; and
- (5) pH Stability
Stable in a pH range of 5.0 to 7.0 when incubated at 4°C for 18 hours;

[0024] Cycloisomaltotetraose-forming enzyme of the present invention, having the above physicochemical properties, may have an amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 6 as the N-terminal amino acid sequence.

[0025] However, cycloisomaltotetraose-forming enzyme having the above physicochemical properties or above N-terminal amino acid sequence is only an example, and enzymes having different physicochemical properties or different

N-terminal amino acid sequences as described above are also encompassed in cycloisomaltotetraose-forming enzyme of the present invention as long as they form cycloisomaltotetraose.

[0026] Usually, cycloisomaltotetraose-forming enzyme of the present invention has a prescribed amino acid sequence. For example, amino acid sequences of SEQ ID NO: 7, SEQ ID NO: 8, or those homologous to SEQ ID NO: 7 or SEQ ID NO: 8 can be listed. A variant enzyme having an amino acid sequence homologous to SEQ ID NO: 7 or SEQ ID NO: 8 means an enzyme having an amino acid sequence where one or more amino acids in SEQ ID NO: 7 or SEQ ID NO: 8 are deleted, replaced or added with other amino acids without altering the enzyme activity of acting on dextran or partial dextran hydrolysates and forming cycloisomaltotetraose. As such a variant enzyme, it is preferable that the enzyme has an amino acid sequence with a homology to SEQ ID NO: 7 or SEQ ID NO: 8 of, usually, 70% or higher, desirably, 80% or higher, more desirably, 90% or higher.

[0027] Although cycloisomaltotetraose-forming enzyme of the present invention is not restricted by its source, microorganisms are preferable as the source. Particularly, a microorganism, strain D1110, isolated by the present inventors from a soil sample or mutants thereof, or a microorganism, strain D2006, also isolated from a soil sample, or mutant thereof can be preferably used. The mutants as referred to as in the present invention means, for example, mutants whose culture properties are improved than the parent strain, enzyme-hyper-producing mutants whose ability of producing cycloisomaltotetraose-forming enzymes are improved than the parent strain, or mutants producing cycloisomaltotetraose-forming enzyme with higher enzymatic activity, created by artificially introducing the mutation into the parent strain.

[0028] As described above, the microorganisms, strain D1110 and strain D2006, having the ability to produce cycloisomaltotetraose-forming enzyme are microorganisms newly isolated from the soil by the present inventors. As described later in Experiment 4, strain D1110 and strain D2006 were identified as *Agreia* sp. and *Microbacterium trichothecenolyticum*, respectively, by identifying the bacterial genus and species based on the homologies of the nucleotide sequences of 16S rRNA (16S rDNA) with those of known bacteria. Based on these results, the present inventors named those novel microorganisms as "*Agreia* sp. D1110" and "*Microbacterium trichothecenolyticum* D2006" and deposited those on December 28, 2018, in National Institute of Technology and Evaluation (NITE), NITE Patent Microorganisms Depository (NPMD), 2-5-6 Kazusakamatari, Kisarazu-shi, Chiba, Japan, and accepted under the accession numbers of NITE BP-02815 and NITE BP-02816, respectively.

[0029] The microorganism having an ability of producing cycloisomaltotetraose-forming enzyme of the present invention includes the above bacterial strains and those mutants, and microorganisms having an ability of producing cycloisomaltotetraose-forming enzyme, belonging other genus and species isolated and selected from nature by the screening comprising the steps of allowing the culture broth as a crude enzyme to act on the substrate dextran and investigating the formation of cycloisomaltotetraose, and those mutants.

[0030] The present invention also relates to the DNAs comprising the nucleotide sequences which encodes the amino acid sequences of cycloisomaltotetraose-forming enzymes of the present invention described above, and complement nucleotide sequences to the nucleotide sequences also described above. The DNA of the present invention includes any DNA originated from the nature and those which are synthesized artificially as far as they encode the amino acid sequence of cycloisomaltotetraose-forming enzyme. For example, microorganisms of the genus *Agreia* including *Agreia* sp. D1110, and microorganisms of the genus *Microbacterium* including *Microbacterium trichothecenolyticum* D2006 can be used as the natural sources of the DNA. A genomic DNA containing the DNA of the present invention can be obtained from the cells of these microorganisms. A genomic DNA containing the DNA can be released extracellularly by the steps of inoculating any of the microorganisms into a nutrient medium, culturing about 5 to 10 days under aerobic conditions, collecting the cells from the culture, and treating the cells with cell-lytic enzymes such as lysozyme and β -glucanase or with ultrasonication. In addition to the methods described above, use of protein-hydrolyzing enzymes such as proteinases, detergents such as SDS, and freeze-thaw method are also applicable. The objective genomic DNA can be obtained from the cells treated by using conventional methods in the art, for example, such as phenol-extraction, alcohol-precipitation, centrifugation, and ribonuclease-treatment. To artificially synthesize the DNA of the present invention, it can be chemically synthesized based on the amino acid sequence described together with the nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10. PCR-method is also applicable to obtain the DNA by using a genomic DNA containing the DNA as a template and an appropriate chemically synthesized DNA as a primer.

[0031] The DNA of the present invention, for example, includes a DNA having a nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10, a DNA having a homologous nucleotide sequence to those, and a DNA having a complement nucleotide sequence to those. A DNA having a homologous nucleotide sequence to SEQ ID NO: 9 or SEQ ID NO: 10, means that having a nucleotide sequence where one or more nucleotides in SEQ ID NO: 9 or SEQ ID NO: 10 are deleted, replaced or added without altering the activity of cycloisomaltotetraose-forming enzyme encoded thereby. The homology (sequence identity) of nucleotide sequence to SEQ ID NO: 9 or SEQ ID NO: 10 of such a variant DNA is preferable to be, usually, 70% or higher, desirably, 80% or higher, more desirably, 90% or higher, and most desirably, 95% or higher. The DNA of the present invention encompasses a DNA having a nucleotide sequence where one or more nucleotides of SEQ ID NO: 9 or SEQ ID NO: 10 are replaced with other nucleotides without altering the encoded amino acid sequence based on the degeneracy of genetic code, and their complement nucleotide sequence.

[0032] A recombinant DNA can be advantageously obtained by inserting the DNA of the present invention into an appropriate autonomously replicable vector. A recombinant DNA is usually composed of an objective DNA and an autonomously replicable vector, and if an objective DNA can be obtained, it can be easily prepared by conventional recombinant DNA techniques. As examples of such vectors, plasmids, phage or cosmid vectors, etc., can be used and appropriately selected depending on the host cell to be introduced or the introduction method. Types of vectors are not specifically restricted, and that possible to be expressed in a host cell can be appropriately selected. Depending on the type of the host cell, a promoter sequence can be appropriately selected to express the gene, and a vector constructed by incorporating the promoter sequence and the gene into various plasmids can be used as the expression vectors. As such expression vectors, for example, phage vectors, plasmid vectors, viral vectors, retroviral vectors, chromosome vectors, episomal vectors and virus-derived vectors (e.g., bacterial plasmid, bacteriophage, yeast episomes, yeast chromosome elements) and viruses (e.g., baculovirus, papovavirus, vaccinia virus, adenovirus, triplex virus, pseudorabies virus, herpes virus, lentivirus and retrovirus) and vectors derived from their combinations (e.g., cosmid and phagemid) are available.

[0033] Preferred vectors for use in bacteria include, for example, pRSET A, pQE-70, pQE-60, pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH6a, pNH18A and pNH46A; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5, and the like. Preferred vectors for use in eukaryotic organisms include, for example, pWLINE0, pSV2CAT, pOG44, pXT1 and pSG; and pSVK3, pBPV, pMSG, pSVL and the like.

[0034] To insert the DNA of the present invention into such vectors, conventional methods used in the field are usually employed. Specifically, the gene DNA containing the objective DNA and an autonomously replicable vector are firstly cleaved by restriction enzymes and/or ultrasonic, then the formed DNA fragment and the vector fragment are ligated. A recombinant DNA thus obtained can be replicated infinitely by the steps of introducing it into an appropriate host and culturing the resulting transformant.

[0035] The recombinant DNA, thus obtained, can be introduced into an appropriate host microorganism including *E. coli*, *Bacillus subtilis*, *Actinomycetes*, yeast, etc. To obtain a transformant, a colony hybridization method can be applied. Also, a transformant can be selected by the steps of culturing the microorganism in nutrient medium and detecting the transformant producing the cycloisomaltotetraose-forming enzyme.

[0036] Any nutrient culture medium can be used for cultivating microorganisms, including a transformant, capable of producing cycloisomaltotetraose-forming enzyme of the present invention as long as they can grow therein and produce cycloisomaltotetraose-forming enzyme: for example, synthetic- and natural-culture media can be used as nutrient culture media. Any carbon source can be used as long as it is utilized for the growth by the microorganisms: examples of such carbon source are saccharides such as dextran and its partial hydrolysates, starch and phytyglycogen, obtainable from plants; glycogen and pullulan, obtainable from animals and microorganisms; hydrolysates thereof, glucose, fructose, lactose, sucrose, mannitol, sorbitol, and saccharide syrups; and organic acids such as citric acid and succinic acid. The concentrations of these carbon sources in nutrient culture media can be appropriately chosen. The nitrogen sources usable for the cultivation are, for example, inorganic nitrogen compounds such as ammonium salts and nitrates; organic nitrogen compounds such as urea, corn steep liquor, casein, peptone, yeast extract and meat extract. The inorganic ingredients usable in the cultivation are, for example, calcium salts, magnesium salts, potassium salts, sodium salts, phosphates, manganese salts, zinc salts, iron salts, copper salts, molybdate salts, and cobalt salts. In addition, if necessary, amino acids and vitamins can be suitably used.

[0037] The microorganisms of the present invention can be cultured under aerobic conditions, usually, at a temperature in the range of 15-37°C and at a pH in the range of 5.5-10, preferably, at a temperature in the range of 20-34°C and at a pH in the range of 5.5-8.5. The cultivation time can be set to a time longer than that required for the growth of the microorganisms, preferably, 10-150 hours. The concentration of dissolved oxygen is not specifically restricted, but usually set to 0.5-20 ppm. The concentration of dissolved oxygen can be kept within the above range by controlling aeration and agitation. The cultivation can be carried out in a batch-wise or a continuous manner.

[0038] After culturing the microorganisms capable of producing cycloisomaltotetraose-forming enzyme, according to the method described above, the culture broth containing the enzyme of the present invention is collected. The major activity of cycloisomaltotetraose-forming enzyme is found in the cell-free supernatant. Both the cell-free supernatant and the culture broth can be used as a crude enzyme. In order to remove cells from the culture broth, conventional liquid-solid separation methods can be used. For example, methods to directly centrifuge the resultant culture broth, as well as those to filtrate the culture broth with pre-coated filters or to separate cells by membrane filtration using plane filters or follow fibers, can be suitably used. Although the cell-free supernatant thus obtained can be used intact as crude enzyme solution, it is generally used after concentration. The concentration methods usable in the invention are, for example, salting out using ammonium sulfate, sedimentation using acetone or alcohol, and concentration using membranes such as plane filters or follow fibers.

[0039] Furthermore, cycloisomaltotetraose-forming enzyme can be subjected to conventional immobilization using the cell-free supernatant and the concentrate. Examples of such conventional methods are conjugation methods using ion exchangers, covalent bindings and adsorptions using resins and membranes, and inclusion methods using high

molecular weight substances.

[0040] As described above, a crude enzyme solution can be used intact or after concentrating it as cycloisomaltotetraose-forming enzyme of the present invention. If necessary, cycloisomaltotetraose-forming enzyme can be advantageously used after separating or purifying the crude enzyme solution by suitable conventional methods used in the art. For example, a purified cycloisomaltotetraose-forming enzyme preparation exhibiting an electrophoretically single band can be obtained by the steps of salting out a cell-free supernatant with ammonium sulfate, dialyzing the resulting crude enzyme preparation, and successively purifying the dialyzed enzyme preparation on anion-exchange column chromatography using "DEAE-TOYOPEARL 650S", an anion-exchange gel commercialized Tosoh Corporation, Tokyo, Japan, and hydrophobic chromatography using "PHENYL-TOYOPEARL 650M", a hydrophobic gel commercialized Tosoh Corporation, Tokyo, Japan.

[0041] When cycloisomaltotetraose-forming enzyme is a recombinant enzyme, the enzyme may be accumulated intracellularly, depending on the kinds of host microorganisms. In such case, while the cells or the culture broth can be used intact, the recombinant enzyme can be advantageously used after extracting it from cells by using osmotic-shock methods or detergents, or by disrupting cells using ultrasonication methods or cell-wall digesting enzymes, and separating it from the cells or cell debris.

[0042] By using the native or recombinant cycloisomaltotetraose-forming enzyme obtained by the above method, it is possible to produce cycloisomaltotetraose of the present invention, and a saccharide composition containing cycloisomaltotetraose.

[0043] In producing cycloisomaltotetraose and a saccharide composition containing cycloisomaltotetraose, α -1,6 glucans such as dextran and partial dextran hydrolysate obtainable by partially hydrolyzing dextran using acids or dextran-hydrolyzing enzyme, dextranase, can be used as a substrate material for the reaction of cycloisomaltotetraose-forming enzyme. Further, as described later in Experiment 14, since cycloisomaltotetraose-forming enzyme of the present invention acts on isomaltotetraose and forms cycloisomaltotetraose, it is revealed that α -1,6 glucans having a glucose polymerization degree of at least 4 can be used as substrates of the enzyme. Furthermore, since the branched α -glucan disclosed in WO2008/136331 pamphlet has an α -1,6 glucan structure that D-glucose molecules are bound *via* α -1,6 linkages as the partial structure, it can be used as a substrate material for the action of cycloisomaltotetraose-forming enzyme.

[0044] When cycloisomaltotetraose-forming enzyme is allowed to act on a substrate, the substrate concentration is not specifically restricted. For example, when using an aqueous substrate solution with a relatively high concentration of 10 % (w/w) or more, the reaction by cycloisomaltotetraose-forming enzyme of the present invention is difficult to proceed, therefore, the substrate concentration of 5 % (w/w) or lower is preferred, and under this condition, cycloisomaltotetraose can be advantageously produced. The reaction temperature used in the present enzymatic reaction can be set to a temperature at which the reaction proceeds, i.e. a temperature up to about 55°C, preferably, a temperature in the range of 30 to 45°C. The reaction pH is controlled in the range of, usually, 4.0 to 8.0, preferably, 4.5 to 7.5. Since the amount of enzyme and the reaction time are closely related, the conditions are adequately chosen with respect to the progress of the objective enzymatic reaction.

[0045] As described above, cycloisomaltotetraose-forming enzyme of the present invention forms cycloisomaltotetraose from α -1,6 glucan having a glucose polymerization degree of 4 or higher, dextran, or a saccharide having α -1,6 glucan structure as a partial structure. Therefore, cycloisomaltotetraose can be produced by using starch or partial starch hydrolysate as a substrate material when cycloisomaltotetraose-forming enzyme of the present invention and an enzyme, having an activity of forming α -1,6 glucan having a glucose polymerization degree of 4 or higher, dextran, or a saccharide having α -1,6 glucan structure as a partial structure from starch or partial starch hydrolysates, are used in combination. For example, dextrin dextranase can be used as the enzyme that acts on starch or partial starch hydrolysates to form α -1,6 glucan having a glucose polymerization degree of 4 or higher and dextran, and an α -glucosyltransferase disclosed in WO2008/136331 pamphlet can be used as the enzyme having an activity of forming a saccharide having α -1,6 glucan structure as a partial structure.

[0046] In producing cycloisomaltotetraose from starch or partial starch hydrolysates by a combination of the enzymes described above, a sequential reaction of firstly forming α -1,6 glucans having a glucose polymerization degree of 4 or higher, dextran, or saccharides having an α -1,6 glucan structure as a partial structure by allowing an enzyme which acts on starch or partial starch hydrolysates and forms α -1,6 glucans having a glucose polymerization degree of 4 or higher, dextran, or saccharides having an α -1,6 glucan structure as a partial structure to act on starch or partial starch hydrolysates, and successively forming cycloisomaltotetraose by allowing cycloisomaltotetraose-forming enzyme of the present invention to act on the resulting products, can be selected. Also, a simultaneous reaction of allowing an enzyme which acts on starch or partial starch hydrolysates and forms α -1,6 glucans having a glucose polymerization degree of 4 or higher, dextran, or saccharides having an α -1,6 glucan structure as a partial structure to act on starch or partial starch hydrolysates and cycloisomaltotetraose-forming enzyme of the present invention to act on starch or partial starch hydrolysates simultaneously, can be selected.

[0047] The reaction mixture, thus obtained by the above reaction, can be used intact as a saccharide solution comprising

cycloisomaltotetraose. Optionally, the saccharide solution comprising cycloisomaltotetraose can be used after hydrolyzing the concomitant isomaltooligosaccharides by allowing dextranase, α -glucosidase, or glucoamylase to act on the saccharide comprising cycloisomaltotetraose. Further, the saccharide comprising cycloisomaltotetraose can be arbitrarily processed by hydrogenating to convert reducing saccharides remained in the reaction mixture into sugar alcohols for eliminating the reducing power. Usually, a saccharide solution comprising cycloisomaltotetraose is used after further purification. Conventional methods used for purifying saccharides can be arbitrarily selected as the purification method. For example, one or more purification methods selected from the group consisting of decoloring with an activated charcoal; desalting with ion exchange resins in H and OH form; fractionation by column chromatography such as ion exchange column chromatography, charcoal column chromatography, and silica gel column chromatography; separation using organic solvents such as alcohol and acetone; separation using a membrane having a suitable separability; fermentation using microorganisms, which utilize and decompose concomitant saccharides but not utilize cycloisomaltotetraose, such as yeasts; and eliminating the remaining reducing sugars with alkaline treatments; can be arbitrarily used.

[0048] More particularly, ion exchange column chromatography can be suitably used as an industrial-scale preparation of the objective saccharides. The objective cycloisomaltotetraose or a saccharide composition comprising the same with an improved purity can be advantageously prepared by, for example, column chromatography using a strong acidic cation exchange resin as described in Japanese Patent Kokai Nos. 23,799/83 and 72,598/83 to remove concomitant saccharides. In this case, any one of fixed bed, moving bed, and semi-moving bed methods can be employed.

[0049] A solution comprising cycloisomaltotetraose thus obtained or a saccharide solution with an improved cycloisomaltotetraose content contains cycloisomaltotetraose in an amount of, usually, 10% (w/w) or higher, desirably, 40% (w/w) or higher, on a dry solid basis (hereinafter, abbreviated as "d.s.b."), and is usually concentrated into a product in a syrupy form. The syrupy product can be arbitrarily dried to make into an amorphous solid product or an amorphous powder product.

[0050] Furthermore, by purifying the aqueous saccharide solution comprising cycloisomaltotetraose by conventional methods, a cycloisomaltotetraose high-content saccharide and a cycloisomaltotetraose preparation with a high purity can be obtained. In addition, crystals of cycloisomaltotetraose and a particulate composition comprising crystalline cycloisomaltotetraose can be prepared by the crystallization using the cycloisomaltotetraose high-content saccharide or the cycloisomaltotetraose preparation with a high purity as a material.

[0051] In addition, cycloisomaltotetraose of the present invention is only slightly hydrolyzed by small intestinal mucosal α -glucosidase. Therefore, cycloisomaltotetraose is a hardly digestible saccharide that not digested and adsorbed when orally ingested, and is considered to be a saccharide with no toxicity and no harm.

[0052] Cycloisomaltotetraose of the present invention and the saccharide compositions comprising the same can be advantageously used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, color-deterioration preventing agent, excipient, base agent for powderization, etc., for various compositions such as foods and beverages, luxury grocery items, feeds, baits, cosmetics, pharmaceuticals, and industrial products in combination with other ingredients.

[0053] Cycloisomaltotetraose of the present invention and the saccharide compositions comprising the same can be used intact as a seasoning for sweetening products. If necessary, they can be advantageously used in combination with other sweeteners, for example, powdery syrup, glucose, isomerized sugar, sucrose, maltose, trehalose, honey, maple sugar, sorbitol, maltitol, dihydrochalcone, stevioside, α -glycosyl stevioside, sweetener of *Momordica grosvenori*, glycyrrhizin, thaumatin, sucralose, L-aspartyl L-phenylalanine methyl ester, saccharine, glycine and alanine; and fillers such as dextrin, starch, and lactose.

[0054] Further, powdery products of cycloisomaltotetraose of the present invention and the saccharide compositions comprising the same can be arbitrarily used intact or, if necessary, after mixing with fillers, excipients, binders, etc., and then shaped into various shapes such as granules, spheres, sticks, plates, cubes, and tablets.

[0055] Cycloisomaltotetraose of the present invention and the saccharide compositions comprising the same have sweetness which well harmonizes with other materials having sour-, salty-, astringent-, *umami*-, and bitter-taste; and cycloisomaltotetraose of the present invention has a high acid- and heat-tolerance as described later in Experiments. Thus, they can be advantageously used to sweeten and/or improve the taste and quality of general foods and beverages.

[0056] Cycloisomaltotetraose of the present invention and the saccharide compositions comprising the same can be advantageously used as a sweetener, taste-improving agent, and quality-improving agent for various seasonings such as a soy sauce, powdered soy sauce, *miso*, "*funmatsu-miso*" (a powdered *miso*), "*moromi*" (a refined sake), "*hishio*" (a refined soy sauce), "*furikake*" (a seasoning powder for rice), mayonnaise, dressing, vinegar, "*sanbai-zu*" (a sauce of sugar, soy sauce and vinegar), "*funmatsu-sushi-zu*" (powdered vinegar for sushi), "*chuka-no-moto*" (an instant mix for Chinese dish), "*tentsuyu*" (a sauce for Japanese deep fat fried food), "*mentsuyu*" (a sauce for Japanese vermicelli), sauce, catsup, "*yakiniku-no-tare*" (a sauce for Japanese grilled meat), curry roux, instant stew mix, instant soup mix, "*dashi-no-moto*" (an instant stock mix), mixed seasoning, "*mirin*" (a sweet sake), "*shin-mirin*" (a synthetic *mirin*), table sugar, and coffee sugar. Also, cycloisomaltotetraose and the saccharide compositions comprising the same can be advantageously used to sweeten and to improve the taste and quality of various "*wagashi*" (Japanese confectionaries)

such as "*senbei*" (a rice cracker), "*arare*" (a fried rice cake cube), "*okoshi*" (a millet and rice cake), "*gyuhi*" (a starch paste), "*mochi*" (a rice cake) and the like, "*manju*" (a bun with a bean-jam), "*uiro*" (a sweet rice jelly), "*an*" (a bean-jam) and the like, "*yokan*" (a sweet jelly of beans), "*mizu-yokan*" (a soft azuki-bean jelly), "*kingyoku*" (a kind of *yokan*), jelly, pao de Castella, and "*amedama*" (a Japanese toffee); Western confectioneries such as a bun, biscuit, cracker, cookie, pie, pudding, butter cream, custard cream, cream puff, waffle, sponge cake, doughnut, chocolate, chewing gum, caramel, nougat, and candy; frozen desserts such as an ice cream and sherbet; syrups such as a "*kajitsu-no-syrup-zuke*" (a preserved fruit) and "*korimitsu*" (a sugar syrup for shaved ice); pastes such as a flour paste, peanut paste, and fruit paste; processed fruits and vegetables such as a jam, marmalade, "*syrup-zuke*" (fruit pickles), and "*toka*" (candied fruits); pickles and pickled products such as a "*fukujin-zuke*" (red colored radish pickles), "*bettara-zuke*" (a kind of whole fresh radish pickles), "*senmai-zuke*" (a kind of sliced fresh radish pickles), and "*rakkyo-zuke*" (pickled shallots); premix for pickles and pickled products such as a "*takuan-zuke-no-moto*" (a premix for pickled radish), and "*hakusai-zuke-no-moto*" (a premix for fresh white rape pickles); meat products such as a ham and sausage; products of fish meat such as a fish ham, fish sausage, "*kamaboko*" (a steamed fish paste), "*chikuwa*" (a kind of fish paste), and "*tenpura*" (a Japanese deep-fat fried fish paste); "*chinmi*" (relish) such as a "*uni-no-shiokara*" (salted urchin), "*ika-no-shiokara*" (salted squid), "*su-konbu*" (vinegared kelp), "*saki-surume*" (dried squid strips), "*fugu-no-mirin-boshi*" (a dried *mirin*-seasoned swellfish), seasoned fish flour such as of Pacific cod, sea bream, shrimp, etc.; "*tsukudani*" (foods boiled down in soy sauce) such as those of laver, edible wild plants, dried squid, small fish, and shellfish; daily dishes such as a "*nimame*" (cooked beans), potato salad, and "*konbu-maki*" (a sea tangle roll); milk products; canned and bottled products such as those of meat, fish meat, fruit, and vegetable; alcoholic beverages such as a synthetic *sake*, fermented liquor, *sake*, fruit liquor, low-malt beer and beer; soft drinks such as a coffee, cocoa, juice, carbonated beverage, sour milk beverage, and beverage containing a lactic acid bacterium; instant food products such as instant pudding mix, instant hot cake mix, instant juice, instant coffee, "*sokuseki-shiruko*" (an instant mix of *azuki-bean* soup with rice cake), and instant soup mix; and other foods and beverages such as solid foods for babies, foods for therapy, drinks, peptide foods, and frozen foods.

[0057] Cycloisomaltotetraose and the saccharide compositions comprising the same can be arbitrarily used to improve the taste preference of feeds and pet foods for animals and pets such as domestic animals, poultry, honey bees, silk worms, and fishes; and also they can be advantageously used as a sweetener and taste-improving agent, taste-curing agent, quality-improving agent, and stabilizer for various compositions including luxury grocery items, cosmetics, pharmaceuticals, quasi drugs in a solid, paste or liquid form such as tobacco, cigarette, tooth paste, lipstick, rouge, lip cream, internal liquid medicine, tablet, troche, cod-liver oil in the form of drop, oral refrigerant, cachou, and gargle.

[0058] When used as a quality-improving agent or stabilizer, cycloisomaltotetraose and the saccharide compositions comprising the same can be advantageously used in biologically active substances susceptible to lose their effective ingredients and activities, as well as in health foods, functional foods, and pharmaceuticals containing the biologically active substances. Example of such biologically active substances are liquid preparations containing lymphokines such as α -, β -, and γ -interferons, tumor necrosis factor- α (TNF- α), tumor necrosis factor- β (TNF- β), macrophage migration inhibitory factor, colony-stimulating factor, transfer factor, and interleukin 2; liquid preparations containing hormones such as insulin, growth hormone, prolactin, erythropoietin, and follicle-stimulating hormone; liquid biological preparations such as BCG vaccine, Japanese encephalitis vaccine, measles vaccine, live polio vaccine, small pox vaccine, tetanus toxoid, Trimeresurus antitoxin, and human immunoglobulin; liquid preparations containing antibiotics such as penicillin, erythromycin, chloramphenicol, tetracycline, streptomycin, and kanamycin sulfate; liquid preparations containing vitamins such as thiamin, riboflavin, L-ascorbic acid, cod liver oil, carotenoid, ergosterol, tocopherol; highly unsaturated fatty acids and their derivatives such as EPA, DHA and arachidonic acid; solution of enzymes such as lipase, esterase, urokinase, protease, β -amylase, isoamylase, glucanase, and lactase; extracts such as ginseng extract, "*suppon*" (soft-shelled turtle) extract, chlorella extract, aloe extract and propolis extract; healthy foods containing biologically active substances such as virus, living microorganisms paste of lactic acid bacteria and yeast, and royal jelly. By using cycloisomaltotetraose and the saccharide compositions comprising the same, the above biologically active substances can be arbitrarily prepared in health foods, functional foods, and pharmaceuticals in a liquid, paste, or solid form, which have a satisfactorily-high stability and quality with less fear of losing or inactivating their effective ingredients and activities.

[0059] The methods for incorporating cycloisomaltotetraose or the saccharide composition comprising the same into the aforesaid compositions are those which can incorporate cycloisomaltotetraose and the saccharide compositions into the compositions before completion of their processing, and which can be appropriately selected from the following conventional methods; mixing, kneading, dissolving, melting, soaking, penetrating, dispersing, applying, coating, spraying, injecting, crystallizing, and solidifying. The amount of cycloisomaltotetraose or the saccharide compositions comprising the same to be preferably incorporated into the final compositions is usually in an amount of 0.1% or higher, desirably, 1% or higher.

[0060] The following experiments explain the present invention in detail.

Experiment 1Products formed from dextran5 Experiment 1-1Preparation of crude enzyme

10 **[0061]** A liquid culture medium (pH 6.8) consisting of 15 g/L of "DEXTRAN T70", a commercially available dextran commercialized by Pharmacosmos A/s., Denmark, 1.0 g/L "POWDERY YEAST EXTRACT SH" a yeast extract commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, 5.0 g/L of "HIPOLYPEPTONE", a polypeptone commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, 1.0 g/L of dipotassium phosphate, 0.6 g/L of sodium phosphate dihydrate, 0.5 g/L of magnesium sulfate hepta-hydrate, 0.01 g/L of ferrous sulfate hepta-hydrate, 0.01 g/L of manganese sulfate penta-hydrate, 3.0 g/L of calcium carbonate and water was placed in twenty 500 mL-Erlenmeyer flasks in respective amounts of 100 mL, sterilized by autoclaving at 121°C for 20 min, and cooled. Successively, the culture medium was inoculated with a microorganism, strain D1110, and followed by cultivation under rotary-shaking conditions at 27°C and 240 rpm for 4 days. After completion of the cultivation, the culture supernatant was obtained by centrifuging the culture broth at 12,000 rpm for 10 minutes to remove cells. To about 0.9 L of the resulting culture supernatant, ammonium sulfate was admixed and dissolved to give 80%-saturation to precipitate proteins. The resulting precipitate was dissolved in 20 mM Tris-HCl buffer (pH 7.5), dialyzed against the same buffer at 4°C for 24 hours, and then centrifuged at 12,000 rpm for 10 min at 4°C. The resulting supernatant was collected as a crude enzyme solution.

Experiment 1-225 Action of the crude enzyme on dextran

30 **[0062]** One liter of 50 mM acetate buffer (pH 6.0) containing 1% (w/v) of "DEXTRAN T70", dextran commercialized by Pharmacosmos A/S, Denmark, and 0.006% (w/v) of hinokitiol as preservative was admixed with the crude enzyme solution obtained in Experiment 1-1, and reacted at 40°C for 20 hours. The reaction mixture was heated at 100°C for 10 min to inactivate the enzyme, concentrated using an evaporator, and dissolved in 100 mL of deionized water. Successively, the resulting solution was admixed with 200 mL of ethanol to precipitate high-molecular weight substances remaining in the reaction mixture such as dextran and proteins, and then the mixture was centrifuged and at 12,000 rpm for 20 min at 4°C, and the resulting supernatant was collected. The resulting supernatant was further subjected to membrane filtration using a membrane with a molecular weight cutoff of 10,000 to remove remaining high-molecular weight substances, and the resulting filtrate was collected as an enzymatic reaction product.

35 **[0063]** To examine saccharides in the resulting enzymatic reaction product, the product was subjected along with isomaltotoligosaccharides markers (a preparation produced by Hayashibara Co., Ltd.), a standard product of isomaltotriose (a preparation produced by Hayashibara Co., Ltd.) and a standard product of isomaltotetraose (a preparation produced by Hayashibara Co., Ltd.) to thin layer chromatography (hereinafter, abbreviated as "TLC") to be developed twice using "KEISEL GEL 60" (aluminum plate, 10 × 20 cm, commercialized by Merck Co., Ltd.) as a thin-layer plate, and a solvent mixture (2-propanol: 1-butanol: water (12: 3: 4, v/v/v)) for development to separate the saccharides in the product. The saccharides separated on the TLC plate were detected by coloring by methanol-sulfuric acid method. The results are shown in FIG 1. As shown in FIG. 1, in comparison with the relative flow rate values (R_f values) of isomaltotoligosaccharide markers (lane 1), the standard product of isomaltotriose (lane 2), and the standard product of isomaltotetraose (lane 3), developed at the same time, a spot of an unknown saccharide (symbol "I" in FIG. 1) in which the R_f value is lower than that of isomaltotriose having a glucose polymerization degree of 3 (symbol "IG3" in FIG. 1) and larger than that of isomaltotetraose having a glucose polymerization degree of 4 (symbol "IG4" in FIG. 1) was detected in lane 4 that the enzyme reaction products were separated. The unknown saccharide was named to "Unknown Saccharide I".

50 Experiment 1-3Preparation of Unknown Saccharide I

55 **[0064]** In the preliminary test using a small amount of the reaction product, Unknown Saccharide I, since it was confirmed that Unknown Saccharide I is not hydrolyzed by α -glucosidase and glucoamylase, and not decomposed by alkaline treatment, subsequently, an experiment for hydrolyzing the dextran hydrolysates concomitant in Unknown Saccharide I to D-glucose. The enzymatic reaction product containing Unknown Saccharide I, obtained in Experiment 1-2, was adjusted to pH 5.0, admixed with 100 U/g-solid of "TRANSGLUCOSIDASE L Amano", α -glucosidase produced by

Amano Enzyme Co., Ltd., and 1,000 U/g-solid of glucoamylase commercialized by Nagase ChemteX Co., Ltd., and then reacted at 50°C for 4 days. After the reaction, the reaction was stopped by heating the reaction mixture at about 100°C for 10 min. The saccharides in the resulting reaction mixture were analyzed by analytical high-performance liquid chromatography (hereinafter, abbreviated as "analytical HPLC"). As a result, D-glucose and Unknown Saccharide I were detected at elution times of 58.7 min and 49.4 min, respectively. The saccharide composition of the reaction mixture is 30.0% (w/w) of D-glucose, 65.5% (w/w) of Unknown Saccharide I, and 4.5% (w/w) of partial dextran hydrolysates containing isomaltooligosaccharides.

[0065] The analytical HPLC was carried out under the following conditions;

Column: "MCI gel CK04SS", produced by Mitsubishi Chemical Corporation, Tokyo, Japan; two columns were connected in series;

Eluent: water;

Column temperature: 80°C;

Flow rate: 0.4 mL/min;

Detector: "RID-10A", a differential refractometer produced by Shimadzu Corporation, Kyoto, Japan.

[0066] Subsequently, reducing saccharides (D-glucose and small amount of partial dextran hydrolysates) in the reaction mixture were decomposed by the steps of admixing sodium hydroxide with the reaction mixture obtained by α -glucosidase and glucoamylase treatment to adjust the pH to 12, and incubating at 98°C for one hour. After removing precipitates by filtration from the resulting solution, the resulting filtrate was admixed with "DIAION SK-1B", a cation exchanger resin produced by Mitsubishi Chemical Corporation, Tokyo, Japan, for neutralizing and adjusting the pH to 8, and then decolorized by activated charcoal treatment. The resulting saccharide solution was further deionized using "DIAION SK-1B", "DIAION WA30", anion exchanger resin produced by Mitsubishi Chemical Corporation, Tokyo, Japan, and "IRA411" anion exchanger resin produced by Organo Corporation, Tokyo, Japan, filtrated by fine filtration, concentrated in an evaporator, and dried in vacuum. As a result, about 60 mg-dry solid of the purified preparation of Unknown Saccharide I was obtained. The saccharide composition of the resulting Unknown Saccharide I preparation was examined by analytical HPLC. As shown in HPLC chromatogram in FIG. 2, the purity of Unknown Saccharide I is 99.1% (w/w) and it was found to be a very high purity preparation.

Experiment 2

Structural analyses of Unknown Saccharide I

[0067] The purified preparation of Unknown Saccharide I, obtained in Experiment 1, was subjected to analyses by LC/MS and various NMR to determine the structure.

Experiment 2-1

Analysis by LC/MS

[0068] The mass of Unknown Saccharide I was measured by subjecting the purified preparation of Unknown Saccharide I obtained in Experiment 1 to "PROMINENCE", a LC/MS analyzer commercialized by Shimadzu Corporation, Kyoto, Japan, using the same LC conditions with HPLC in the above Experiment 1-3. In the HPLC, the peak of Unknown Saccharide I was eluted at the retention time of 49.5 min, and sodium-added molecular ion with a mass number (m/z) of 671 was significantly detected. Accordingly, it was revealed that the mass of Unknown Saccharide I is 648. The value of the mass suggested that Unknown Saccharide I is a cyclic gluco-tetrasaccharide having a structure that four D-glucose molecules are bound to give a cyclic form.

Experiment 2-2

Analyses by ^1H -NMR and ^1H - ^1H COSY

[0069] In order to determine the chemical structure of Unknown Saccharide I, the purified preparation of Unknown Saccharide I obtained in Experiment 1 was dissolved in heavy water (D_2O), and subjected to nuclear magnetic resonance (NMR) analyses, ^1H -NMR and ^1H - ^1H -COSY, using "AVANCE II Spectrometer", a NMR apparatus commercialized by Bruker-Japan, Kanagawa, Japan. The ^1H -NMR spectrum and the ^1H - ^1H COSY spectrum of Unknown Saccharide I are shown in FIG. 3 and FIG. 4, respectively.

[0070] As shown in FIG. 3, seven main peaks were observed in the ^1H -NMR spectrum of Unknown Saccharide I (Since

heavy water was used as a solvent for NMR analysis, protons of OH groups were not detected in the spectrum). From the integration ratio of the peak, it was found that seven protons with different environments are present in the molecule of Unknown Saccharide I. The doublet peak observed at 5.13 ppm was assigned to the C-1 proton bound to anomeric carbon of D-glucose. Based on the ^1H - ^1H COSY spectrum shown in FIG. 4, peaks were sequentially assigned based on the correlation with the C-1 proton. The results of the assignments of the seven proton peaks are shown in Table 1.

Table 1

Chemical shift (ppm)	Coupling constant (Hz)	Splitting pattern	Assignment
5.13	3.9	d	1H
3.42	9.9, 3.8	dd	2H
3.64	9.5	t	3H
3.14	9.5	t	4H
4.08	10.4	t	5H
4.05	11.3, 2.6	dd	6Ha
3.64	10.7	t	6Hb
d: Doublet dd: Double doublet t: Triplet 1H-6H: Protons bound to carbons at C1-C6 positions of D-glucose			

[0071] From the values of the coupling constant and chemical shift of each proton peak shown in Table 1, it was found that Unknown Saccharide I has a glucopyranosyl structure, and the binding mode of D-glucoses of Unknown Saccharide I are α -anomer because the peak of C-1 proton bound to the anomeric carbon was detected at 5.13 ppm.

Experiment 2-3

Analyses by ^{13}C -NMR, HSQC, and HMBC

[0072] Using the NMR apparatus used in Experiment 2-2, Unknown Saccharide I was subjected to ^{13}C -NMR, HSQC (Heteronuclear Single-Quantum Correlation Spectroscopy) and HMBC (Heteronuclear Multiple Bond Correlation Spectroscopy) analyses. ^{13}C -NMR, HSQC, and HMBC spectra of Unknown Saccharide I are shown in FIG. 5, FIG. 6, and FIG. 7, respectively.

[0073] As shown in FIG. 5, only six carbon peaks were observed in the ^{13}C -NMR spectrum of Unknown Saccharide I. From the molecular mass obtained by LC/MS analysis in Experiment 2-1, it was suggested that Unknown Saccharide I is a cyclic gluco-tetrasaccharide having a structure of binding four D-glucose molecules to form cyclic structure. Since only six carbon peaks were observed, it was suggested that Unknown Saccharide I is a cyclic gluco-tetrasaccharide constructed by a single binding mode. Carbon observed at 95.522 ppm in the ^{13}C -NMR spectrum was assigned to anomeric carbon.

[0074] Based on the results of the assignment of the peaks detected in ^1H -NMR spectrum shown in Table 1, the carbon showing correlation with each proton in the HSQC spectrum shown in FIG. 6 were assigned and the results were shown in Table 2.

Table 2

Chemical shift (ppm)	Assignment
95.5	1C
71.2	2C
72.2	3C
71.9	4C
68.8	5C
67.4	6C

[0075] In the HMBC spectrum shown in FIG. 7, carbons showing a correlation with the C-1 proton were investigated. As the results, C-2, C-3, C-5, and C-6 carbons showed correlation with C-1 proton, and C-4 carbon does not show correlation. Since C-1 proton showed a correlation between C-6 carbon in addition to C-2, C-3, and C-5 carbons, it was revealed that in Unknown Saccharide I, glucosidic linkage is present between C-1 carbon and C-6 carbon, i.e., D-glucoses in Unknown Saccharide I are bound via 1,6-glucosidic linkages.

[0076] From the above results, it was revealed that Unknown Saccharide I is a cyclic glucotetrasaccharide having a structure of binding four D-glucose molecules to form a cyclic structure via α -1,6 glucoside linkages, i.e., cycloisomaltotetraose, as shown in FIG. 8. Since a saccharide having such a structure has not been known at all, cycloisomaltotetraose is a novel cyclic saccharide.

Experiment 3

Crystal of cycloisomaltotetraose

[0077] The preparation of the crystal of cycloisomaltotetraose was tried using a purified preparation of cycloisomaltotetraose (25.9 g, purity: 96.1% (w/w)) re-prepared by scaling-up the method in Experiment 1.

Experiment 3-1

Preparation of crystal

[0078] Crystals were precipitated by the step of suspending the purified preparation of cycloisomaltotetraose in deionized water; heating the resulting suspension to dissolve the saccharide; adjusting the saccharide concentration (Brix value) of the resulting cycloisomaltotetraose solution to 53.2 %; and cooling the solution to room temperature. A micrograph of the crystals is shown in FIG. 9. The precipitated crystals were collected by filtration and dried under a reduced pressure at 25°C, and then, 8.6 g of crystalline preparation of cycloisomaltotetraose was obtained. The purity of cycloisomaltotetraose of the crystals was determined by analytical HPLC described above and the value was high as 99.5% by weight, d.s.b.

Experiment 3-2

Analyses of crystalline cycloisomaltotetraose

[0079] The crystalline preparation of cycloisomaltotetraose obtained in Experiment 3-1 was subjected to a measurement of the moisture content analysis, thermal analysis, and powder X-ray diffraction analysis using the conventional methods.

Moisture content

[0080] The moisture content of the crystalline preparation of cycloisomaltotetraose obtained in Experiment 3-1 was measured using "AVQ-2200A", a Karl-Fischer moisture measuring apparatus commercialized by Hiranuma Sangyo Co., Ltd., Ibaraki, Japan, and the value of 13.9% by weight was obtained. The theoretical value of the moisture content when assuming the crystalline cycloisomaltotetraose is pentahydrate crystal or hexahydrate crystal is 12.2% by weight or 14.3% by weight. Accordingly, the cycloisomaltotetraose crystal was considered to be either pentahydrate crystal or hexahydrate crystal.

Thermal Analysis (DSC, TG/DTA)

[0081] About 5 mg of the crystalline preparation of cycloisomaltotetraose, obtained in Experiment 3-1, was weighed into an aluminum container and subjected to thermal analysis using "TG/DTA 6200", a differential heat weight simultaneous measuring apparatus commercialized by Hitachi High-Tech Science Corporation, Tokyo, Japan, with heating the container from 30°C to 300°C at a heating rate of 5°C/min under a nitrogen atmosphere. As a result, weight loss of 12.12% by weight was observed up to 116.5°C. The weight loss was considered to be due to desorbing of crystalline water. Considering the result with that for moisture content, combined with the above findings for moisture content, the crystalline cycloisomaltotetraose was considered to be pentahydrate crystal.

Powder X-ray Diffraction Pattern

[0082] Powder X-ray diffraction analysis of crystalline cycloisomaltotetraose pentahydrate was carried out by placing the sample on a Si-reflection free sample holder and using "X' PERT PRO MPD", a powder X-ray (CuK α -ray) diffraction apparatus commercialized by Spectris Co., Ltd., Tokyo, Japan. The powder X-ray diffraction diagram of crystalline cycloisomaltotetraose pentahydrate is shown in FIG. 10. Characteristic diffraction peaks were observed at diffraction angles (2 θ) of 9.0°, 9.8°, 11.8°, 13.1° and 19.1° (Symbols "↓" in FIG. 10).

Experiment 4Identification of microorganisms capable of producing cycloisomaltotetraose-forming enzyme

[0083] In a screening from soil, two novel microorganisms, D1110 and D2006 were isolated, and D2006 showed a different colony form from D1110. By the microscopic observation, it was found that both microorganisms, D1110 and D2006, are rod shaped bacteria. In this experiment, species of the microorganisms, D1110 and D2006, were identified by determining the nucleotide sequences of those 16S rRNA (16S rDNA).

Experiment 4-1Preparation of 16S rDNA from microorganisms, D1110 and D2006

[0084] A medium (pH 6.8), containing 1.5 g/L of "DEXTRAN T70", commercially available dextran commercialized by Pharmacosmos A/S, Denmark, 1.0 g/L of dipotassium phosphate, 1.0 g/L of magnesium sulfate heptahydrate, 1.0 g/L of sodium chloride, 2.0 g/L of ammonium sulfate, 0.01 g/L of ferrous sulfate heptahydrate, 0.01 g/L of manganese sulfate heptahydrate, 0.001 g/L of zinc sulfate heptahydrate, 20.0 g/L of gellan gum, and water, was sterilized by autoclaving at 121°C for 20 min, and then, the medium was poured into a shale and cooled to prepare gellan gum plate medium. Successively, microorganisms, D1110 and D2006, isolated from soil, were respectively inoculated into the plate medium, cultured at 27°C for 3 days for allowing to form single colonies.

[0085] Single colonies of D1110 and D2006 obtained above were respectively taken and suspended in 50 μ L of "MIGHTYPREP REAGENT FOR DNA", a commercially available DNA simple extraction reagent commercialized by Takara Bio Inc., Shiga, Japan, and treated at 95°C for 10 min. After the treatment, the solution was centrifuged at 15,000 rpm for 2 min for collecting supernatant containing genomic DNA. Each genomic DNA of D1110 and D2006 was subjected to PCR using a sense primer, "8F", having a nucleotide sequence of SEQ ID NO: 1 and an antisense primer, "1512R", having a nucleotide sequence of SEQ ID NO: 2, and the resulting PCR-amplified product was subjected to agarose gel electrophoresis. Since a PCR-amplified product with a length of about 1.5 kbp was observed respectively, the products were collected by ethanol precipitation and used as 16S rDNA.

Experiment 4-2Determination of the nucleotide sequence of 16S rDNA

[0086] Each nucleotide sequence of 16S rDNA from D1110 and D2006, obtained in Experiment 4-1, was determined by a conventional method, respectively. It was revealed that 16S rDNAs from D1110 and D2006 have nucleotide sequences of SEQ ID NO: 3 (1,447 bp) and SEQ ID NO: 4 (1,485 bp), respectively.

Experiment 4-3Identification of microorganisms, D1110 and D2006, by homology search of those 16S rDNA

[0087] Based on the nucleotide sequence of 16S rDNA determined in Experiment 4-2, homology search was carried out for the databases RDP (The Ribosomal Database Project) and EzTaxon, using "GENETYX Ver. 13", a gene analysis software.

[0088] As the results of the homology search for 16S rDNA from D1110 obtained in Experiment 4-2, the genus, species, and strain, and the value of homology to their 16S rDNA nucleotide sequences of the top 5 among the top 20 entries are shown in Table 3. Further, the results of the homology search for 16S rDNA from D2006 obtained by the same method are also shown in Table 4.

Table 3

Rank	Name	Strain	Accession No.	Homology(%)
1	<i>Agreia Pratensis</i>	VKM Ac-2510(T)	jgi.1118336	98.34
2	<i>Agreia bicolorate</i>	VKM Ac-1804(T)	JYFC01000015	98.20
3	<i>Subtercola boreus</i>	K300(T)	AF224722	97.28
4	<i>Leifsonia antarctica</i>	SPC-20(T)	AM931710	97.23
5	<i>Herbiconiux ginsengi</i>	CGMCC 4.3915(T)	jgi.1076294	97.10

Table 4

Rank	Name	Strain	Accession No.	Homology(%)
1	<i>Microbacterium trichothecenolyticum</i>	DSM 8608	JY JA0100006	99.17
2	<i>Microbacterium hibisci</i>	THG-T2.14	KX456190	98.46
3	<i>Microbacterium jejuense</i>	THG-C31	JX997974	98.38
4	<i>Microbacterium yannicii</i>	G72	FN547412	98.34
5	<i>Microbacterium testaceum</i>	DSM 20166	X77445	98.20

[0089] As shown in Table 3, the nucleotide sequence of 16S rDNA of D1110 showed the highest homology of 98.34% between that of *Agreia pratensis* VKM Ac-2510 (T). Generally, the classification of bacteria based on the 16S rDNA nucleotide sequence is recognized to be likely the same species if there is a homology of 99% or higher. Since there is no nucleotide sequence showing the homology of 99% or higher in the search, it could not lead to the identification of species. However, it was revealed that D1110 belongs to the genus *Agreia*. Based on the result, D1110 was identified to *Agreia* sp., and named to *Agreia* sp. D1110.

[0090] Further, as shown in Table 4, the nucleotide sequence of 16S rDNA of D2006 showed the highest homology of 99.17% between that of *Microbacterium trichothecenolyticum* DSM8608. Based on this result, the genus and species of D2006 was identified as *Microbacterium trichothecenolyticum*, and D2006 was named to *Microbacterium trichothecenolyticum* D2006.

[0091] Both *Agreia* sp. D1110 and *Microbacterium trichothecenolyticum* D2006 were deposited to the National Institute of Technology and Evaluation (NITE) Patent Microbiology Depository Center (NPMD), respectively, and accepted on December 28, 2018 under the accession Nos. NITE BP-02815 and NITE BP-02816, respectively.

Experiment 5

Preparation of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815)

[0092] The same liquid medium used in Experiment 1-1 was placed by 3mL in the test tube, sterilized by autoclaving at 121°C for 20 minutes, and cooled. Then, *Agreia* sp. D1110 (NITE BP-02815) was inoculated and cultured at 27°C, for 96 hours with shaking at 240 rpm to obtain a seed culture.

[0093] To 20 Erlenmeyer flasks with a 500 mL-volume, 100 mL each of the same liquid medium for the seed culture was dispensed, and the medium was sterilized by autoclaving at 121 °C for 20 min and cooled to 27°C. Then, 1 mL each of the seed culture obtained above was inoculated to each liquid culture medium and cultured by rotary-shaking at 27°C for 66 hours. After the cultivation, the culture broth was centrifuged at 12,000 rpm for 15 min to remove cells and about 1.8 L of culture supernatant was obtained. The culture supernatant was subjected to assay for cycloisomaltotetraose-forming enzyme activity, and it was revealed that cycloisomaltotetraose-forming enzyme activity of the culture supernatant was 0.112 unit/mL.

Experiment 6

Purification of enzyme from *Agreia* sp. D1110 (NITE BP-02815)

[0094] About 1.8 L of the culture supernatant (Total activity: 202 units) obtained in Experiment 5 was salted out by adding ammonium sulfate to give finally 80% saturation and allowing it to stand at 4°C for 24 hours. The resultant precipitates were collected by centrifuging at 12,000 rpm for 30 min, dissolved in 20 mM Tris-HCl buffer (pH 7.5), and dialyzed against the same buffer to obtain about 153 mL of a dialyzed enzyme solution. The dialyzed enzyme solution had 33.2 units of cycloisomaltotetraose-forming activity. The enzyme solution was then subjected to anion-exchange column chromatography using 24.1 mL of "DEAE-TOYOPEARL 650S" gel, an anion-exchange gel commercialized by Tosoh Corporation, Tokyo, Japan. Cycloisomaltotetraose-forming enzyme activity was adsorbed on "DEAE-TOYOPEARL 650S" gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted by 0 M-0.5 M NaCl linear gradient. The active fractions were eluted at about 0.25 M NaCl, collected and admixed with ammonium sulfate to give a final concentration of 1 M, and then centrifuged to remove precipitates. Successively, the enzyme solution was subjected to hydrophobic column chromatography using 11 mL of "PHENYL-TOYOPEARL 650M" gel, a hydrophobic gel commercialized by Tosoh Corporation, Tokyo, Japan. Cycloisomaltotetraose-forming enzyme activity was adsorbed on "PHENYL-TOYOPEARL 650M" gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 M of ammonium sulfate and when eluted with a linear gradient decreasing from 1 M to 0 M of ammonium sulfate, cycloisomaltotetraose-forming enzyme activity was eluted at about 0.3 M of ammonium sulfate. The amount of total enzyme activity, total protein, specific activity and yield of cycloisomaltotetraose-forming enzyme in each purification step are shown in Table 5.

Table 5

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture supernatant	202.0	440	0.46	100
Dialyzed solution after salting out with ammonium sulfate	33.2	260	0.13	17
Eluate from DEAE-TOYOPEARL 650S column chromatography	21.8	25	0.88	11
Eluate from PHENYL-TOYOPEARL 650M column chromatography	20.3	2.5	8.1	10

[0095] The purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by final hydrophobic chromatography using "PHENYL-TOYOPEARL 650M" gel, was assayed for purity on SDS-polyacrylamide gel electrophoresis using a 8-16% (w/v) gradient gel and detected on the gel as a single protein band, i.e., a high purity preparation.

Experiment 7

Properties of enzyme from *Agreia* sp. D1110 (NITE BP-02815)

Experiment 7-1

Molecular weight

[0096] The purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 6, was subjected to SDS-polyacrylamide gel electrophoresis (a 6 to 16% (w/v) gradient gel) and molecular weight of cycloisomaltotetraose-forming enzyme was measured comparing with those of "PRECISION PLUS UNCOLORED PROTEIN STANDARD", a molecular weight markers commercialized by Bio-Rad Japan, Tokyo, Japan, also subjected to the electrophoresis at the same time. It was revealed that cycloisomaltotetraose-forming enzyme has a molecular weight of $86,000 \pm 10,000$ daltons.

Experiment 7-2Optimum temperature and optimum pH for the enzyme reaction

[0097] Effects of temperature and pH on the enzyme activity were investigated using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 6, by varying temperature and pH at the assay of the enzyme. The results are shown in FIG. 11 (Optimum temperature) and FIG. 12 (Optimum pH), respectively. It was revealed that the optimum temperature of cycloisomaltotetraose-forming enzyme is 35 to 40°C when reacted at pH 6.0 for 30 min and the optimum pH is 5.5 to 6.0 when reacted at 40°C for 30 min.

Experiment 7-3Thermal and pH stabilities of the enzyme

[0098] Thermal stability and pH stability of the enzyme were investigated using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 6. Thermal stability of the enzyme was determined by the steps of incubating an enzyme solution (20 mM acetate buffer, pH 6.0) under various temperatures for 30 min, cooling in water, and measuring the residual enzyme activity. pH Stability of the enzyme was determined by the steps of incubating enzyme solution in 100 mM buffer at various pHs and at 4°C for 16 hours, adjusting the pH to 6.0, and measuring the residual enzyme activity. The results are shown in FIG. 13 (Thermal stability) and in FIG. 14 (pH Stability), respectively. As is evident from the results in FIG. 13, cycloisomaltotetraose-forming enzyme is stable up to 40°C. As is evident from the results in FIG. 14, it was revealed that cycloisomaltotetraose-forming enzyme is stable in the range of pH 5.0 to 10.0.

Experiment 7-4N-Terminal (NH₂-Terminal) amino acid sequence

[0099] The N-terminal amino acid sequence of the enzyme from D1110 was determined using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 6, by using "Model PPSQ-31A", a protein sequencer commercialized by Shimadzu Corporation, Kyoto, Japan. As a result, it was revealed that the enzyme has the N-terminal amino acid sequence of SEQ ID NO: 5, i.e., Ala-Thr-Val-Gly-Asp-Ala-Trp-Thr-Asp-Lys-Ala-Arg-Tyr-Asp-Pro.

Experiment 8Preparation of enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816)

[0100] The same liquid medium used in Experiment 1-1 was placed by 3mL in the test tube, sterilized by autoclaving at 121°C for 20 min, and cooled. Then, *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) was inoculated and cultured at 27°C for 96 hours with shaking at 240 rpm to obtain a seed culture.

[0101] To 15 Erlenmeyer flasks with a 500 mL-volume, 100 mL each of the same liquid medium for the seed culture was dispensed, and the medium was sterilized by autoclaving at 121°C for 20 min and cooled to 27°C. Then, 1 mL each of the seed culture obtained above was inoculated to each liquid culture medium and cultured by rotary-shaking at 27°C for 66 hours. After the cultivation, the culture was centrifuged at 12,000 rpm for 15 min to remove cells and about 1.4 L of culture supernatant was obtained. The culture supernatant was subjected to assay for cycloisomaltotetraose-forming enzyme activity, and it was revealed that cycloisomaltotetraose-forming enzyme activity of the culture supernatant was 0.069 unit/mL.

Experiment 9Purification of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816)

[0102] About 1.4 L of the culture supernatant (Total activity: 96.6 units) obtained in Experiment 8 was salted out by adding ammonium sulfate to give finally 80% saturation and allowing it to stand at 4°C for 16 hours. The resultant precipitates were collected by centrifuging at 12,000 rpm for 30 min, dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM calcium chloride, and dialyzed against the same buffer to obtain about 110 mL of a dialyzed enzyme solution. The

dialyzed enzyme solution had 140.7 units of cycloisomaltotetraose-forming activity. The enzyme solution was subjected to anion-exchange column chromatography using 20 mL of "DEAE-TOYOPEARL 650S" gel, an anion-exchange gel commercialized by Tosoh Corporation, Tokyo, Japan. Cycloisomaltotetraose-forming enzyme activity was adsorbed on "DEAE-TOYOPEARL 650S" gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM calcium chloride and eluted with a linear gradient from 0 M to 0.5 M of NaCl. The active fractions eluted at about 0.2 M NaCl was collected and dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM calcium chloride. Successively, the dialyzed enzyme solution was subjected to anion-exchange column chromatography using 1.0 mL of "Mono Q 5/50GL" gel, a gel commercialized by GE Healthcare Japan, Tokyo, Japan. Cycloisomaltotetraose-forming enzyme activity was adsorbed on "Mono Q 5/50GL" gel equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM calcium chloride and when eluted with a linear gradient from 0 M to 0.5 M of NaCl, cycloisomaltotetraose-forming enzyme activity was eluted at about 0.3 M NaCl. The amount of total enzyme activity, total protein, specific activity and yield of cycloisomaltotetraose-forming enzyme in each purification step are shown in Table 6.

Table 6

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture supernatant	96.6	387.8	0.25	100
Dialyzed solution after salting out with ammonium sulfate	140.7	143.1	0.98	146
Eluate from DEAE-TOYOPEARL 650S column chromatography	50.0	31.9	1.57	51.3
Eluate from PHENYL-TOYOPEARL 650M column chromatography	10.5	2.95	3.55	10.9

[0103] The purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by hydrophobic chromatography using "Mono Q 5/50GL" gel, was assayed for purity on SDS-polyacrylamide gel electrophoresis (a 8-16% (w/v) gradient gel) and detected on the gel as a single protein band, i.e., a high purity preparation.

Experiment 10

Properties of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816)

Experiment 10-1

Molecular weight

[0104] The purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 9, was subjected to SDS-polyacrylamide gel electrophoresis (a 6-16% (w/v) gradient gel) and molecular weight of cycloisomaltotetraose-forming enzyme was measured comparing with "PRECISION PLUS UNCOLORED PROTEIN STANDARD", a molecular weight markers commercialized by Bio-Rad Japan, Tokyo, Japan, also subjected to the electrophoresis at the same time. It was revealed that cycloisomaltotetraose-forming enzyme has a molecular weight of $86,000 \pm 10,000$ daltons.

Experiment 10-2

Optimum temperature and optimum pH for the enzyme reaction

[0105] Effects of temperature and pH on the enzyme activity were investigated using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 9, by varying temperature and pH at the assay of the enzyme. The results are shown in FIG. 15 (Optimum temperature) and FIG. 16 (Optimum pH), respectively. It was revealed that the optimum temperature of cycloisomaltotetraose-forming enzyme is 40°C when reacted at pH 6.0 for 30 min and the optimum pH is around 5.5 when reacted at 40°C for 30 min.

Experiment 10-3Thermal and pH stabilities of the enzyme

[0106] Thermal stability and pH stability of the enzyme were investigated using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 9. Thermal stability of the enzyme was determined by the steps of incubating an enzyme solution (20 mM acetate buffer, pH 6.0) under various temperatures for 30 min, cooling in water, and measuring the residual enzyme activity. pH Stability of the enzyme was determined by the steps of incubating enzyme solution in 100 mM buffer at various pHs, and at 4°C for 18 hours, adjusting the pH to 6.0, and measuring the residual enzyme activity. The results are shown in FIG. 17 (Thermal stability) and in FIG. 18 (pH Stability), respectively. As is evident from the results in FIG. 17, cycloisomaltotetraose-forming enzyme is stable up to 35°C. As is evident from the results in FIG. 18, it was revealed that cycloisomaltotetraose-forming enzyme is stable in the range of pH 5.0 to 7.0.

Experiment 10-4N-Terminal amino acid sequence

[0107] The N-terminal amino acid sequence of the enzyme from D2006 was determined using the purified enzyme preparation of cycloisomaltotetraose-forming enzyme, obtained by the method in Experiment 9, by using "Model PPSQ-31A", a protein sequencer commercialized by Shimadzu Corporation, Kyoto, Japan. As a result, it was revealed that the enzyme has the N-terminal amino acid sequence of SEQ ID NO: 6, i.e., Ala-Asp-Leu-Gly-Asp-Ala-Trp-Thr- Asp.

Experiment 11Cloning of a DNA encoding cycloisomaltotetraose-forming enzyme and preparation of a recombinant DNA comprising the DNA and a transformant

[0108] DNAs encoding cycloisomaltotetraose-forming enzyme were respectively cloned from *Agreia* sp. D1110 (NITE BP-02815) and *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), and self-replicable recombinant DNAs containing the DNA were constructed. Successively, the nucleotide sequences of the DNAs encoding the enzyme were determined and transformants were also prepared.

Experiment 11-1Preparation of genomic DNAs

[0109] Cells of *Agreia* sp. D1110 (NITE BP-02815) or *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), subcultured on agar medium plate, was taken in an amount of one loop from the plate, inoculated to 3 mL of the liquid medium used in Experiment 1 in a test tube, and cultured under shaking conditions at 27°C and 240 rpm for 5 days. After completion of the cultivation, cells were collected by centrifuging the culture broth, and genomic DNA was prepared by a conventional method using "DNeasy Blood & Tissue Kit", a commercially available whole DNA purification kit, commercialized by QIAGEN N. V., Netherland.

Experiment 11-2Determination of the nucleotide sequence of whole genome using a next-generation sequencer

[0110] Each genomic DNA from respective microorganism, obtained in Experiment 11-1, was enzymatically fragmented using "Nextera XT DNA Library Preparation Kit", a commercially available kit commercialized by Illumina K. K., Tokyo, Japan, and the resulting DNA fragments were treated to have blunt ends, added the adaptor sequences, and made into a library. After amplifying the library by PCR, the amplified library was purified by using "AMPure XP", a commercially available DNA purification kit commercialized by Beckman Coulter Inc., USA. Then, the nucleotide sequences of the DNA fragments in the library were determined by using "MiSeq", a next-generation sequencer commercialized by Illumina K. K., Tokyo, Japan, and the nucleotide sequence of the whole genomic DNA was obtained by integrating the above nucleotide sequences (contig sequences) using a computer.

[0111] Successively, the nucleotide sequence of the whole genomic DNA was analyzed by using "Glimmer", a gene region-predicting software and open reading frames (ORF: deduced gene region) being estimated to encode proteins

were predicted. As the results, it was found that the whole genomic DNA of *Agreia* sp. D1110 (NITE BP-02815) has 9,139 ORFs and that of *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) has 11,068 ORFs.

Experiment 11-3

Identification of an ORF encoding cycloisomaltotetraose-forming enzyme

[0112] An ORF encoding an amino acid sequence that matches with the N-terminal amino acid sequence of cycloisomaltotetraose-forming enzyme, determined in Experiment 3-2, (SEQ ID NO: 5) was searched from 9,139 ORFs of the whole genomic DNA from *Agreia* sp. D1110 (NITE BP-02815), detected in whole genome analysis in Experiment 11-2. As the result, it was found that an amino acid sequence identical to the N-terminal amino acid sequence was encoded in ORF9038. From the result, it was found that the nucleotide sequence of ORF9038, i.e., the nucleotide sequence of SEQ ID NO: 9 is that of the structural gene DNA of the cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815), and also found that the cycloisomaltotetraose-forming enzyme has an amino acid sequence that N-terminal amino acid sequence consisting of 31 amino acids deduced to secretion signal sequence was deleted from the amino acid sequence described together with the nucleotide sequence of SEQ ID NO: 9, i.e., amino acid sequence of SEQ ID NO: 7.

[0113] An ORF encoding an amino acid sequence that matches with the N-terminal amino acid sequence of cycloisomaltotetraose-forming enzyme determined in Experiment 10-4, (SEQ ID NO: 6) was searched from 11,068 ORFs of the whole genomic DNA from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), detected in whole genome analysis in Experiment 11-2. As the result, it was found that an amino acid sequence identical to the N-terminal amino acid sequence was encoded in ORF5328. From the result, it was found that the nucleotide sequence of ORF5328, i.e., the nucleotide sequence of SEQ ID NO: 10 is that of the structural gene DNA of the cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), and also found that the cycloisomaltotetraose-forming enzyme has an amino acid sequence that N-terminal amino acid sequence consisting of 32 amino acids deduced to secretion signal sequence was deleted from the amino acid sequence described together with the nucleotide sequence of SEQ ID NO: 10, i.e., amino acid sequence of SEQ ID NO: 8.

[0114] The molecular weight of the cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815) having the amino acid sequence of SEQ ID NO: 7 was calculated to be 86,350, and that of the enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) having the amino acid sequence of SEQ ID NO: 8 was calculated to be 86,658. These values were well matched with the molecular weights of $86,000 \pm 10,000$ obtained by SDS-PAGE in Experiments 3-1 and 10-1, respectively. The homology (sequence identity) between the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 9, that of the enzyme gene from *Agreia* sp. D1110 (NITE BP-02815) and the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 10, that of the enzyme gene from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) was investigated by using "GENETYX Ver. 13", a commercially available genetic information processing software commercialized by Genetyx Corporation, Tokyo, Japan, and it was calculated to be 71% (see FIG. 19).

Experiment 12

Preparation of a recombinant DNA for expression, pRSET A-ORF9038 and production of a recombinant cycloisomaltotetraose-forming enzyme by its transformant, E9038

[0115] The gene encoding cycloisomaltotetraose-forming enzyme in ORF9038 was amplified from genomic DNA from *Agreia* sp. D1110 (NITE BP-02815) by conventional PCR method and inserted to "pRSET A", an expression vector commercialized by Thermo Fisher Scientific K. K., Tokyo, Japan, and the expression of a recombinant cycloisomaltotetraose-forming enzyme in *E. coli* was investigated.

Experiment 12-1

Preparation of a recombinant DNA for expression, pRSET A-ORF9038 and a transformant, E9038

[0116] In order to insert the gene encoding cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815) to "pRSET A", an expression vector, an amplified DNA fragment was obtained by PCR against the whole genomic DNA of *Agreia* sp. D1110 (NITE BP-02815) using a sense primer having a nucleotide sequence of SEQ ID NO: 11 and an anti-sense primer having a nucleotide sequence of SEQ ID NO: 12 in combination. The PCR amplified DNA was designed to have nucleotide sequences of 15 bases homologous to pRSET A at both 5'- and 3'-ends for inserting it into the vector, pRSET A, by In-fusion method, to place the DNA encoding the amino acid sequence of the mature cyclois-

omaltotetraose-forming enzyme, i.e., amino acid sequence of SEQ ID NO: 7, just after initiation codon ATG, and to place termination codon TAA TAG just after the DNA. T7 promoter, ribosome-binding site (RBS) and initiation codon ATG were used those present on pRSET A.

[0117] A recombinant DNA, obtained by inserting the above amplified DNA into the vector pRSET A by In-fusion method, was named to "pSET A-ORF9038". pRSET A-ORF-9038 is shown in FIG. 20. Then, *E. coli* HST-08, commercialized by Takara Bio Inc., Shiga, Japan, was transformed by using pSET A-ORF9038, and pRSET A-ORF9038 was isolated from the resulting transformant. Further, a host *E. coli* BL21 (DE3) for expression, commercialized by Merk Japan, was transformed by using the isolated pRSET A-ORF9038 to obtain a transformant, "E9038".

Experiment 12-2

Production of a recombinant cycloisomaltotetraose-forming enzyme by the transformant, E9038

[0118] A liquid TB medium (pH 6.8) consisting of 47 g/L of "TERRIFIC BROTH" commercialized by Thermo Fisher Scientific K. K., Tokyo, Japan, 4 g/L of glycerol and water was placed in a glass tube in respective amount of 5 mL, sterilized by autoclaving at 121°C for 20 min, and cooled. Then, ampicillin was aseptically admixed into the liquid medium to give a final concentration of 100 µg/mL. The transformant, E9038, obtained by the method in Experiment 12-1, was inoculated into the above liquid medium, and cultured at 37°C for 8 hours under a shaking condition, and then admixed with isopropyl-1-thio-β-D-galactopyranoside (IPTG) to give a final concentration of 100 µM for inducing the expression of a gene encoding cycloisomaltotetraose-forming enzyme, and the cultivation was further continued for 16 hours. Cells were collected from the culture broth by centrifugation. According to the conventional methods, cells were subjected to lysozyme treatment, freeze-thaw treatment, and ultrasonic disruption to obtain cell extract. The ultrasonic disruption was carried out by suspending cells in 20 mM Tris-HCl buffer (pH 7.5) and disrupting cells in suspension in an ice bath using a ultrasonic homogenizer, "Model UH-600", commercialized by MST Corporation, Aichi, Japan, and the resulting cell extract was centrifuged to obtain supernatant as a cell-free extract.

[0119] The cell-free extract thus obtained was used as a crude enzyme solution of the recombinant cycloisomaltotetraose-forming enzyme, and 0.3 mL of the crude enzyme solution was admixed with 0.3 mL of 200 mM acetate buffer (pH 6.0) containing 2.0% (w/v) of "DEXTRAN T70", a dextran commercialized by Pharmacosmos A/s., Denmark. After incubating the resulting mixture at 40°C for overnight, the mixture was heated at 100°C for 10 min to stop the reaction. The reaction mixture thus obtained was subjected to the analytical HPLC used in Experiment 1. The HPLC chromatogram is in FIG. 21. As a control, the *E. coli* BL21 (DE3) introduced with the expression vector, pRSET A, was cultured under the same conditions as in the case of transformants described above, to prepare a cell-free extract from the culture, and then the cycloisomaltotetraose-forming activity was examined by the same manner.

[0120] As shown in FIG. 21, in the HPLC chromatogram of the reaction mixture, a peak of dextran (symbol "Dex" in FIG. 21) used as substrate was eluted at the retention time of about 17 min, and a peak of cycloisomaltotetraose (symbol "CI-4" in FIG. 21) was detected at the retention time of about 49.4 min. From the result, it was qualitatively confirmed that cycloisomaltotetraose-forming enzyme gene is expressed in cells of the host *E. coli* and cycloisomaltotetraose-forming enzyme is produced. Although a concrete chromatogram was omitted, the formation of cycloisomaltotetraose was not detected in the case of using the cell-free extract from the control host *E. coli*, and the enzyme activity could not be detected at all.

[0121] Although the details were omitted, also in the case of the enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), the recombinant DNA and transformant were prepared and the recombinant enzyme was expressed in cells of host *E. coli* in the same manner of the above enzyme from *Agreia* sp. D1110 (NITE BP-02815), and as the result, the production of cycloisomaltotetraose-forming enzyme was detected.

Experiment 13

Action of cycloisomaltotetraose-forming enzyme on isomaltopentaose

[0122] As a part of examining the substrate specificity of cycloisomaltotetraose-forming enzyme, the action of the enzyme on isomaltopentaose was investigated. To 0.5 mL of 50 mM acetate buffer (pH 6.0) containing 1% (w/v) of isomaltopentaose (prepared in Hayashibara Co., Ltd., Okayama, Japan, purity: 98.1% by weight) and 0.006% (w/v) hinokitiol as a preservative, 2.0 units/g-isomaltopentaose of a purified cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815), obtained by the method in Experiment 6, was added, and the resulting mixture was incubated at 40°C for 24 hours and then heated at 100°C for 10 min to inactivate the enzyme. To examine the reaction products in the reaction mixture, saccharides were separated by TLC using "KIESELGEL 60", a TLC aluminum plate (10 x 20 cm) and a solvent (acetonitrile/water/ethyl acetate/1-propanol, volume ratio of 85:70:20:50) and two-times ascending method. The separated saccharides on the plate were detected by visualizing the spots with sulfate-methanol method,

and the reaction products formed from isomaltopentaose were investigated.

[0123] As the result, it was found that cycloisomaltotetraose-forming enzyme acts on isomaltopentaose and produces cycloisomaltotetraose with isomaltotetraose, also slightly isomaltotriose, isomaltose, glucose and high molecular glucans. From this result, it was considered that the enzyme acts on α -1,6 glucans with a glucose polymerization degree of at least 5 or higher.

Experiment 14

Substrate specificity of cycloisomaltotetraose-forming enzyme

[0124] Substrate specificity of cycloisomaltotetraose-forming enzyme was investigated using various saccharides as substrates. Aqueous solutions containing D-glucose, maltose, isomaltose, panose, isomaltotriose, isomaltotetraose, isomaltopentaose, isomaltohexaose, isomaltoheptaose, dextran T10 (average molecular weight: 10,000, commercialized by Pharmacosmos A/s., Denmark), dextran T70 (average molecular weight: 70,000, commercialized by Pharmacosmos A/s., Denmark), or dextran T2000 (average molecular weight: 2,000,000, commercialized by Pharmacosmos A/s., Denmark) were prepared as substrate solutions. To each substrate solution, acetate buffer and calcium chloride were added to give final concentrations of 50 mM and 1 mM, respectively, and then resulting solutions were admixed with 20 units/g-solid of cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815), obtained by the method in Experiment 6, adjusted the concentration to 1% (w/v) and incubated at pH 6.0, 40°C for 24 hours. To examine the saccharides in each reaction mixture before and after the reaction, saccharides were separated by silica gel thin-layer chromatography (hereinafter, abbreviated as "TLC") using "KIESELGEL 60F₂₅₄", a TLC aluminum plate (10 x 20 cm) and a solvent (2-propanol/1-butanol/water, volume ratio of 12:3:4) with two-times ascending method. The separated saccharides on the plate were detected by visualizing the spots with sulfate-methanol method, and the degree of forming cycloisomaltotetraose from each substrate and the products other than cycloisomaltotetraose were investigated. The results are shown in Table 7.

Table 7

Substrate	Formation of cycloisomaltotetraose
D-Glucose	-
Maltose	-
Isomaltose	-
Panose	-
Isomaltotriose	-
Isomaltotetraose	+
Isomaltopentaose	++
Isomaltohexaose	+++
Isomaltoheptaose	+++
Dextran (Average molecular weight: 10,000)	+++
Dextran (Average molecular weight: 70,000)	+++
Dextran (Average molecular weight: 2,000,000)	+++
... - : Not formed + : Slightly formed ++ : Formed +++ : Significantly formed	

[0125] As shown in Table 7, among the tested saccharides, cycloisomaltotetraose-forming enzyme did not act on monosaccharide, disaccharides, and trisaccharides, such as D-glucose, maltose, isomaltose, panose, and isomaltotriose. While, the enzyme acted on isomaltotetraose, isomaltopentaose, isomaltohexaose, isomaltoheptaose, and dextrans and formed cycloisomaltotetraose. From the results, it was found that the enzyme acts on α -1,6 glucans with a glucose polymerization degree of 4 or higher and forms cycloisomaltotetraose. The enzyme formed isomaltotetraose by hydrolyzing the formed cycloisomaltotetraose. Further, it was found that the enzyme acts on α -1,6 glucans with a glucose

polymerization degree of 4 or higher and catalyzes disproportionation reaction of forming a series of isomaltooligosaccharides with a glucose polymerization degree of 2 or higher. In addition, the enzyme formed isomaltohexaose when allowed to act on cycloisomaltotetraose in the presence of isomaltose as an acceptor. From the result, it was found that the enzyme catalyzes a coupling reaction of hydrolyzing cycloisomaltotetraose and transferring the formed isomaltotetraose to isomaltose. In the same manner, substrate specificity of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) was also investigated, and it was found that the enzyme also acts on α -1,6 glucans with a glucose polymerization degree of 4 or higher and forms cycloisomaltotetraose as in the case of the enzyme from *Agreia* sp. D1110 (NITE BP-02815). Further, the enzyme was same with that from D1110 in the points of catalyzing hydrolysis of cycloisomaltotetraose, disproportionation of α -1,6 glucans, and coupling reaction. Overall, the substrate specificity of the enzyme was equivalent to the enzyme from D1110.

Experiment 15

Digestivity test of cycloisomaltotetraose using small intestinal mucosal enzyme

[0126] The crystalline cycloisomaltotetraose preparation with a purity of 99.5% by weight, obtained in Experiment 3-1, was dissolved in 50 mM maleic acid buffer (pH 6.0) to give a concentration of 1% by weight to make into a substrate solution. To the substrate solution, 50 units/g-cycloisomaltotetraose, as maltose-hydrolyzing activity, of small intestine mucosal enzyme (α -glucosidase) prepared from rat small intestine acetone powder, commercialized by Sigma-Aldrich Co., was added, and the resulting mixture was incubated at 37°C for 24 hours and then heated at 100°C for 10 min to inactivate the enzyme. The amounts of cycloisomaltotetraose in the solution before and after the reaction were determined by HPLC, and the residual rate (%) of cycloisomaltotetraose after the enzymatic reaction, i.e., (the amount after the reaction/the amount before the reaction) x 100, was measured. As a result, the residual rate after 24-hours reaction was 91.6%. From the result, it was revealed that cycloisomaltotetraose is a hardly digestible saccharide being only slightly degraded by small intestinal mucosal enzyme.

Experiment 16

Thermal stability of cycloisomaltotetraose

[0127] An aqueous cycloisomaltotetraose solution with a solid concentration of 7% (w/v) was prepared by dissolving the crystalline cycloisomaltotetraose preparation with a purity of 99.5% by weight, obtained in Experiment 3-1. Eight milliliters each of the aqueous solution was taken in a glass centrifuge tube with a screw cap, sealed, and then heated for 30 to 90 min at 120°C. The solutions before heating and those after heating for various times were subjected to the measurement for the degree of coloring and for the cycloisomaltotetraose purity by HPLC method. The degree of coloring was defined as the absorbance at 480 nm measured by using a 1-cm cell. The results are shown in Table 8.

Table 8

Heating time (min)	Degree of coloring (A480nm)	Purity of cycloisomaltotetraose (% by weight)
0	0.000	99.5
30	0.000	99.5
60	0.000	99.5
90	0.000	99.5

[0128] As is evident from the results in Table 8, aqueous solutions of cycloisomaltotetraose was not colored by heating at a high temperature, 120°C, and purity reduction due to decomposition was not also observed. Accordingly, it was found that cycloisomaltotetraose is a stable saccharide against heating.

Experiment 17

pH Stability of cycloisomaltotetraose

[0129] The crystalline cycloisomaltotetraose preparation obtained in Experiment 3-1 was dissolved in various buffers to make 7 kinds of aqueous cycloisomaltotetraose solutions with a cycloisomaltotetraose concentration of 4% (w/v), and with the pH adjusted to 3 to 9. Eight milliliters each of the aqueous solution was taken into a glass centrifuge tube with

a screw cap, sealed, and heated at 100°C for 24 hours. After cooling them, the measurement for the degree of coloring degree of those aqueous solution and for the cycloisomaltotetraose purity were carried out in the same manner in Experiment 16. The results are shown in Table 9.

Table 9

pH	Buffer	Degree of coloring (A480nm)	Purity of cycloisomaltotetraose (% by weight)
3	Acetate	0.000	93.5
4	Acetate	0.000	99.4
5	Acetate	0.000	99.4
6	Tris-HCl	0.000	99.4
7	Tris-HCl	0.000	99.5
8	Tris-HCl	0.001	99.5
9	Ammonium	0.000	99.4

[0130] As is evident from Table 9, cycloisomaltotetraose was not substantially degraded in the range of pH 4 to 9 by heating at 100°C for 24 hours. Accordingly, it was found that cycloisomaltotetraose is stable in a wide range from weakly acidic to weakly alkaline conditions. However, it was slightly decomposed at pH 3, and a reduction of the purity was observed.

[0131] The following examples explain the present invention in detail. However, the present invention is not restricted by them.

Example 1

[0132] A liquid medium (pH 6.8) containing 15 g/L of "DEXTRAN T70", a commercially available dextran commercialized by Pharmacosmos A/s., Denmark, 1.0 g/L of "POWDERY YEAST EXTRACT SH", a yeast extract commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, 5.0 g/L of "HIPOLYPEPTONE", a polypeptone commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, 1.0 g/L of dipotassium phosphate, 0.6 g/L of sodium phosphate dihydrate, 0.5 g/L of magnesium sulfate heptahydrate, 0.01 g/L of ferrous sulfate hexahydrate, 0.01 g/L of manganese sulfate pentahydrate, 3.0 g/L of calcium carbonate and water was placed in fifty 500 mL-Erlenmeyer flasks in respective amounts of 100 mL, sterilized by autoclaving at 121°C for 20 min, and cooled. Then, *Agreia* sp. D1110 (NITE BP-02815) was inoculated into the liquid medium and cultured by rotary-shaking the flasks at 240 rpm and at 27°C for 4 days. After completion of the cultivation, cells were removed by centrifuging the culture broth at 12,000 rpm for 10 min and culture supernatant was obtained. Then, about 4.8 L of the culture supernatant was concentrated using a UF-membrane and about 1,100 mL of a concentrated enzyme solution, containing 0.70 unit/mL of cycloisomaltotetraose-forming enzyme activity, was collected as a crude enzyme solution.

Example 2

[0133] Except for replacing "POWDERY YEAST EXTRACT SH", a yeast extract commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, to "YEAST EXTRACT MEAST GRANULE S", a yeast extract commercialized by Asahi Food & Healthcare, Ltd., Tokyo, Japan; and "HIPOLYPEPTONE", a polypeptone commercialized by Nihon Pharmaceutical Co., Ltd., Tokyo, Japan, to "HINUTE S", commercialized by Fuji Oil Co., Ltd., Osaka, Japan, a liquid medium (pH 6.8) having the same composition with that in Example 1, was placed in fifty 500 mL-Erlenmeyer flasks in respective amounts of 100 mL, sterilized by autoclaving at 121°C for 20 min, and cooled. Then, *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) was inoculated into the liquid medium and cultured by rotary-shaking the flasks at 240 rpm and at 27°C for 4 days. After completion of the cultivation, cells were removed by centrifuging the culture broth at 12,000 rpm for 10 min and culture supernatant was obtained. Further, about 4.7 L of the culture supernatant was concentrated using a UF-membrane and about 920 mL of a concentrated enzyme solution, containing 0.41 unit/mL of cycloisomaltotetraose-forming enzyme activity, was collected as a crude enzyme solution.

Example 3

[0134] Ten liters of 50 mM acetate buffer (pH 6.0) containing 2% (w/v) of "DEXTRAN T70", dextran commercialized by Pharmacosmos A/s., Denmark, and 0.006% (w/v) of hinokitiol as a preservative was admixed with 5 units/g-dextran

of a crude enzyme solution containing cycloisomaltotetraose-forming enzyme from *Agreia* sp. D1110 (NITE BP-02815), obtained by the method of Example 1, and then incubated at 40°C for 24 hours. The reaction mixture containing 11.2% by weight, d.s.b., of cycloisomaltotetraose and 1.8% by weight, d.s.b., of isomaltotetraose was obtained. After inactivating the enzyme by heating the reaction mixture at 95°C for 30 min and cooling, high molecular substances were removed by filtrating the reaction mixture using a UF membrane with a molecular weight cut off of 10,000, and a saccharide solution (filtrate) containing 62% by weight, d.s.b., of cycloisomaltotetraose and 12.8% by weight, d.s.b., of isomaltotetraose was obtained. The obtained filtrate was purified by conventional methods, decoloring using activated charcoal and deionizing using ion-exchanger resins with H⁺ and OH⁻ forms, and further concentrated, and a syrup with a solid concentration of 60% by weight was obtained. The syrup as a saccharide solution was subjected to a column chromatography using "AMBERLITE CR-1310" (Na-form), a strongly acidic cation-exchange resin commercialized by Organo Corporation, Tokyo, Japan. The resin was packed into four jacketed stainless-steel columns having a diameter of 5.4 cm, which were then cascaded in series to give a total gel bed depth of 20 m. Under the conditions of keeping the inner column temperature at 60°C, the saccharide solution was fed to the columns in a volume of 5% (v/v) and fractionated by feeding to the columns hot water heated to 60°C at an SV (space velocity) of 0.13 to obtain fractions containing cycloisomaltotetraose. While monitoring the saccharide composition of elute by HPLC, and then the fractions containing cycloisomaltotetraose were collected and the fractions were concentrated. As a result, a cycloisomaltotetraose solution with a cycloisomaltotetraose purity of 98.6% by weight, d.s.b., was obtained. Successively, cycloisomaltotetraose was crystallized according to the method described in Experiment 3-1 and crystalline cycloisomaltotetraose pentahydrate with a purity of 99.8% by weight, d.s.b., was obtained. Since the product showed no reducing power, it can be advantageously used in various compositions such as foods and beverages, cosmetics, and pharmaceuticals as a quality-improving agent, stabilizer, excipient, base material for powderization, and the like.

Example 4

[0135] Twenty liters of 50 mM acetate buffer (pH 6.0) containing 2.5% (w/v) of "DEXTRAN T70", dextran commercialized by Pharmacosmos A/s., Denmark, and 0.006% (w/v) of hinokitiol as a preservative was admixed with 5 units/g-dextran of a crude enzyme solution containing cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816), obtained by the method of Example 2, and then incubated at 35°C for 48 hours. The reaction mixture containing 9.7% by weight, d.s.b., of cycloisomaltotetraose and 2.0% by weight, d.s.b., of isomaltotetraose was obtained. After inactivating the enzyme by heating the reaction mixture at 95°C for 30 min and cooling, high molecular substances were removed by filtrating the reaction mixture using a UF membrane with a molecular weight cut off of 10,000, and a saccharide solution (filtrate) containing 62% by weight, d.s.b., of cycloisomaltotetraose and 12.8% by weight, d.s.b., of isomaltotetraose was obtained. The obtained saccharide solution was further concentrated and dried to make into a particulate composition containing cycloisomaltotetraose. The product contained 64% by weight, d.s.b., of cycloisomaltotetraose, 13% by weight, d.s.b., of isomaltotetraose, and 25% by weight, d.s.b., of other saccharides with a glucose polymerization degree of 7 or higher. The product can be advantageously used in various compositions such as foods and beverages, cosmetics, and pharmaceuticals as a quality-improving agent, stabilizer, excipient, base material for powderization, and the like.

Example 5

[0136] A commercially available corn starch was suspended in 20 mM acetate buffer (pH 5.5) containing 1 mM calcium chloride to give a concentration of 30% by weight, dissolved by heating, and then cooled to 50°C. To the resulting material solution, α -glucosyl transferase disclosed in International Patent Application No. WO2008/136331 pamphlet, isoamylase (a starch debranching enzyme), and "KLEISTASE E5CC", an α -amylase commercialized by Amano Enzyme, Aichi, Japan, were respectively admixed to give an enzyme amount of 10 units, 1,000 units, and 10 units per one gram solid of the substrate, and allowed to react at 50°C for 72 hours. Successively, the reaction mixture was heated at 100°C for 15 min to inactivate enzymes and a reaction mixture containing branched α -glucans having α -1,6 glucan as a partial structure was prepared.

[0137] A part of the reaction mixture containing branched α -glucans obtained above was diluted with 100 mM acetate buffer (pH 6.0) to give a concentration of 1.0 % by weight. To the diluted solution, 2.2 units/g-solid substrate of cycloisomaltotetraose-forming enzyme from *Microbacterium trichothecenolyticum* D2006 (NITE BP-02816) was admixed and allowed to react at 40°C for 48 hours. The reaction product contained 8.1% by weight of cycloisomaltotetraose, 5.7% by weight of isomaltotetraose, 4.2% by weight of isomaltotriose, and 5.0% by weight of isomaltose, d.s.b.. From the result, it was found that cycloisomaltotetraose can be produced from starchy substrate by using an enzyme which forms a saccharide having α -1,6 glucan as a partial structure from starch and cycloisomaltotetraose-forming enzyme in combination.

Example 6

Glucosyl derivatives of cycloisomaltotetraose

[0138] As an acceptor and a saccharide donor for a transglycosylation reaction, 0.5 g of crystalline cycloisomaltotetraose pentahydrate, obtained by the method in Example 3, and 2.5 g of "CAVAMAX W6 food", a commercially available α -cyclodextrin commercialized by CycloChem Co., Ltd., Hyogo, Japan, were used, respectively. The acceptor and the saccharide donor were dissolved in 50 mL of 50 mM acetate buffer (pH 5.5) containing 1 mM calcium chloride to make into a substrate solution. The substrate solution was admixed with 2 units/g-saccharide donor of a glucanotransferase, CGTase from *Geobacillus stearothermophilus*, incubated at 50°C for 24 hours, and then heated at 100°C for 10 min to stop the enzyme reaction. Successively, the resulting reaction mixture was admixed with 10 units/g-saccharide donor of "GLUCOZYME #2000", a commercially available glucoamylase commercialized by Nagase ChemteX Corporation, Osaka, Japan, incubated at 50°C for 24 hours, and then heated at 100°C for 10 min to stop the enzyme reaction. To the resulting glucoamylase-treated solution, sodium hydroxide was admixed to adjust the pH to 13.0, and the resulting solution was subjected to alkaline treatment by autoclaving at 105 °C for one hour to degrade reducing sugars in the glucoamylase-treated solution. The resulting alkaline-treated solution was subjected to deionization, concentration and filtration, and a solution containing glucosyl-derivatives of cycloisomaltotetraose was obtained.

[0139] The saccharide solution containing glucosyl derivatives of cycloisomaltotetraose obtained above was subjected to HPLC analysis under the following conditions. The resulting chromatogram is shown in FIG. 22.

(HPLC conditions)

[0140]

Column: YMC-Pack ODS-AQ303 (4. 6 mm x 250 mm) (commercialized by YMC Co., Ltd., Kyoto, Japan);
Eluent: water/methanol (95:5, v/v);
Column temperature: 40°C;
Flow rate: 0. 7 mL/min;
Detection: Refractive index detector

[0141] As shown in FIG. 22, a peak of cycloisomaltotetraose was detected at a retention time of 6.47 min and two peaks considered to glucosyl-derivatives of cycloisomaltotetraose were detected at retention times of 9.12 min and 11.36 min. Further, a peak of α -cyclodextrin used as a saccharide donor was detected at a retention time of 19.1 min and a peak of β -cyclodextrin considered to be formed from α -cyclodextrin was detected at a retention time of 47.7 min. Saccharides eluted at retention times of 9.12 min and 11.36 min were designated to Glucosyl derivatives A and B, respectively. The contents of Glucosyl derivatives A and B in the saccharide solution containing glucosyl derivatives were 31.6% by weight, d.s.b. and 15.8% by weight, d.s.b., respectively.

[0142] The saccharide solution containing glucosyl derivatives was subjected to preparative HPLC using "D-ODS-5", an ODS column commercialized by YMC Co., Ltd., Kyoto, Japan, and Glucosyl derivatives A and B were collected. As the results, 229 mg of Glucosyl derivative A with a purity of 99.2% by weight, d.s.b., and 117 mg of Glucosyl derivative B with a purity of 98.0% by weight, d.s.b., were obtained. The preparations of Glucosyl derivatives A and B were subjected to LC/MS analysis and NMR analysis. From the analyses, it was revealed that the molecular mass of Glucosyl derivative A is 810 and Glucosyl derivative A is a penta-saccharide having a structure that one glucose molecule is bound to one of 4 glucose residues constructing cycloisomaltotetraose via α -1,4 glucosidic linkage, i.e., a mono-glucosyl derivative of cycloisomaltotetraose. It was also revealed that the molecular mass of Glucosyl derivative B is 972 and Glucosyl derivative B is a hexa-saccharide having a structure that two glucose molecules are bound to 2 glucose residues located diagonally in 4 glucose residues constructing cycloisomaltotetraose via α -1,4 glucosidic linkages, i.e., a di-glucosyl derivative of cycloisomaltotetraose. The structures of the glucosyl derivatives of cycloisomaltotetraose, Glucosyl derivatives A and B, are shown in FIG. 23.

Example 7

Hard candy

[0143] Thirty parts by weight of a crystalline cycloisomaltotetraose pentahydrate, obtained by the method in Example 3, was admixed with 100 parts by weight of sucrose solution with a sucrose concentration of 55% (w/v) with heating. Then, the mixture was concentrated under a reduced pressure to give a moisture content of less than 2%. The resulting concentrate was admixed with 0.6 part by weight of citric acid and an appropriate amount of lemon flavor and coloring,

shaped into hard candy according to a conventional method. The product shows a satisfactory crisp, taste, flavor, and hardly causes the crystallization of sucrose due to containing cycloisomaltotetraose. The product is a high-quality hard candy with low hygroscopicity and no fluidity.

5 Example 8

Chocolate cookies

10 **[0144]** Using 140 parts by weight of flour (thin flour), 90 parts by weight of butter, 115 parts by weight of chocolate, 360 parts by weight of granulated sugar, 200 parts by weight of whole egg, 200 parts by weight of almond, and 30 parts by weight of powdery product containing cycloisomaltotetraose, prepared by the method of Example 4, chocolate cookies were produced by a conventional method. After 2 weeks storage at room temperature, the product was tasted. It was found that the product shows no moisture absorption and drying, and further, cycloisomaltotetraose suppresses oxidation and degradation of fats and oils and retained its flavor well right after production.

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Example 9

Lactic acid bacteria beverage

20 **[0145]** One hundred seventy-five parts by weight of skim milk, 30 parts by weight of a powdery product containing cycloisomaltotetraose, obtained by the method in Example 4, and 70 parts by weight of "NYUKA-OLIGO", a lactosucrose high content powder commercialized by Hayashibara Co., Ltd, Okayama, Japan, were dissolved into 1,500 parts by weight of water, and then the resulting mixture was sterilized at 65°C for 30 min. After cooling the mixture to 40°C, 30 parts by weight of a lactic acid bacterium culture was inoculated to the mixture as a starter according to the conventional method, and cultured at 37°C for 8 hours to obtain a lactic acid bacteria beverage. The product has a satisfactory flavor and keeps the lactic acid bacterium stable because it comprises oligosaccharides and cycloisomaltotetraose. Further, the product is preferably used as a lactic acid bacteria beverage having a growth-promoting activity for bifidobacteria and a bowel-regulating property.

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30 Example 10

Body soap

35 **[0146]** Fifteen parts by weight of potassium lauric acid, 5.0 parts by weight of potassium myristate, 4.0 parts by weight of a powdery product containing cycloisomaltotetraose, obtained by the method of Example 4, 2.0 parts by weight of propylene glycol, 0.5 part by weight of polyethylene powder, 0.5 part by weight of hydroxypropyl chitosan solution, 0.25 part by weight of glycine, 0.25 part by weight of glutamine, 0.1 part by weight of photosensitive dye No. 201, an appropriate amount of phenol, pH adjusting agent, and an appropriate amount of lavender water were mixed and then purified water was added to the mixture to give the total amount of 100 parts by weight, and successively a body soap was prepared by emulsifying the mixture by a conventional method. The product is a good foaming body soap with excellent feeling of use. Further, the product can be advantageously used for preventing the generation of body odor, preventing itching, or for the treating or preventing the aging of the skin. Further, the product has an excellent moisturizing property because it contains cycloisomaltotetraose, and it can be used without concern of hypersensitivity because it is less irritating to the skin.

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Example 11

Cosmetic cream

50 **[0147]** According to conventional method, 2 parts by weight of polyoxyethylenglycol mono-stearate, 5 parts by weight of self-emulsified glycerin mono-stearate, 2 parts by weight of a crystalline cycloisomaltotetraose pentahydrate, obtained by the method in Example 3, one part by weight of "αG-RUTIN", α-glucosyl rutin, commercialized by Hayashibara Co. Ltd., Okayama, Japan, one part by weight of liquid paraffin, 10 parts by weight of glycerin-trioctanoate and an appropriate amount of preservative were mixed and dissolved by heating. The resulting mixture was further admixed with 2 parts by weight of L-lactic acid, 5 parts by weight of 1,3-butylen glycol, and 66 parts by weight of purified water, and the resulting mixture was emulsified using a homogenizer. The homogenized mixture was further admixed with an appropriate amount of flavor and stirred to make into a cosmetic cream. The product has an antioxidative activity of α-glucosyl rutin and said antioxidative activity is stabilized by cycloisomaltotetraose, therefore, it can be advantageously used as a

55

sunburn preventive, skin-care agent and whitening agent for skin.

Example 12

Toothpaste

[0148] Forty-five parts by weight of calcium monohydrogen phosphate, 1.5 parts by weight of sodium lauryl sulfate, 25 parts by weight of glycerin, 0.5 part by weight of polyoxyethylene sorbitan laurate, 10 parts by weight of a crystalline cycloisomaltotetraose pentahydrate, obtained by the method in Example 3, 0.02 part by weight of saccharin, and 18 parts by weight of water were mixed to make into a toothpaste. The product is a toothpaste whose bad taste is improved and shows a satisfactory availability without losing the washing power of surfactant.

INDUSTRIAL APPLICABILITY

[0149] According to the present invention, a novel saccharide, cycloisomaltotetraose, having a structure in which four D-glucose molecules are bound in cyclic form via α -1,6 glucosidic linkages, and a saccharide composition comprising the same can be produced and provided by using a cycloisomaltotetraose-forming enzyme. The present invention, which enables to provide a novel cycloisomaltotetraose, contributes to various fields such as foods and beverages, cosmetics, and pharmaceuticals, and its industrial significance is extremely large.

EXPLANATION OF SYMBOLS

[0150]

In FIG. 1,

Lane 1: Isomaltooligosaccharides (marker);

Lane 2: Isomaltotriose (standard product);

Lane 3: Isomaltotetraose (standard product);

Lane 4: Reaction products obtained by allowing a crude enzyme from a microorganism D1110 strain to act on a dextran substrate;

G: Glucose;

IG2: Isomaltose;

IG3: Isomaltotriose;

IG4: Isomaltotetraose;

I: An unknown saccharide;

In FIGs. 4, 6 and 7,

The dashed line is an auxiliary line added by the applicant for signal assignment.

1H: Proton bound to the carbon at C1 position of D-glucose

2H: Proton bound to the carbon at C2 position of D-glucose

3H: Proton bound to the carbon at C3 position of D-glucose

4H: Proton bound to the carbon at C4 position of D-glucose

5H: Proton bound to the carbon at C5 position of D-glucose

6Ha and 6Hb: Protons bound to the carbon at C6 position of D-glucose

In FIGs 6 and 7,

1C: Carbon at C1 position of D-glucose

2C: Carbon at C2 position of D-glucose

3C: Carbon at C3 position of D-glucose

4C: Carbon at C4 position of D-glucose

5C: Carbon at C5 position of D-glucose

6C: Carbon at C6 position of D-glucose

In FIG. 10,

↓: Characteristic diffraction peaks in the powder X-ray diffraction pattern of crystalline cycloisomaltotetraose pen-

tahydrate.

In FIG. 19,

ORF9038: Amino acid sequence encoded by cycloisomaltotetraose-forming enzyme gene derived from *Agreia* sp. D1110.

ORF5328: Amino acid sequence encoded by cycloisomaltotetraose-forming enzyme gene derived from *Microbacterium trichothecenolyticum* D2006.

In FIG. 20,

f1 ori: f1 phage replication origin;

Ampicillin: Ampicillin resistance gene;

pUC ori: pUC replication origin;

P_{T7}: T7 promoter;

RBS: Ribosome binding site;

ATG: Initial codon;

(Black arrow): cycloisomaltotetraose-forming enzyme gene;

In FIG. 21,

Dex: Dextran;

CI-4: Cycloisomaltotetraose;

In FIG. 22,

CI-4: Cycloisomaltotetraose;

A: Cycloisomaltotetraose derivative A;

B: Cycloisomaltotetraose derivative B;

α -CD: α -Cyclodextrin;

β -CD: β -Cyclodextrin;

In FIG. 23

(A) Derivative A: A glucosyl derivative of cycloisomaltotetraose;

(B) Derivative B: A di-glucosyl derivative of cycloisomaltotetraose;

SEQUENCE LISTING

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				500						505					510					
15		ggc	tac	gtc	aac	ctc	gac	agc	gtc	acg	ctg	ggc	act	ttc	gac	aca	ccc	1584		
		Gly	Tyr	Val	Asn	Leu	Asp	Ser	Val	Thr	Leu	Gly	Thr	Phe	Asp	Thr	Pro			
				515					520					525						
		tca	gta	cag	ctc	gcc	aac	gcc	gcg	ttc	gcg	gcg	aac	ggg	gca	tcg	cac	1632		
		Ser	Val	Gln	Leu	Ala	Asn	Ala	Ala	Phe	Ala	Ala	Asn	Gly	Ala	Ser	His			
				530				535					540							
20		atc	gag	atg	ggc	cag	ggc	gac	cag	atg	ctc	gtc	gcg	ccc	tac	ttc	ctc	1680		
		Ile	Glu	Met	Gly	Gln	Gly	Asp	Gln	Met	Leu	Val	Ala	Pro	Tyr	Phe	Leu			
				545			550					555					560			
		gac	gaa	acc	aag	cag	atg	aac	cac	gaa	ctg	caa	ggg	tgg	ttg	gag	aag	1728		
		Asp	Glu	Thr	Lys	Gln	Met	Asn	His	Glu	Leu	Gln	Gly	Trp	Leu	Glu	Lys			
				565							570					575				
		tac	tac	gac	gtc	atc	acc	ggc	tac	gag	aac	ctc	ttc	tac	ggc	ccc	acc	1776		
		Tyr	Tyr	Asp	Val	Ile	Thr	Gly	Tyr	Glu	Asn	Leu	Phe	Tyr	Gly	Pro	Thr			
				580						585					590					
		ctc	cgt	cag	cta	tcg	aac	gcc	gtg	acc	atc	agc	ggt	caa	ccg	aca	agc	1824		
		Leu	Arg	Gln	Leu	Ser	Asn	Ala	Val	Thr	Ile	Ser	Gly	Gln	Pro	Thr	Ser			
				595				600						605						
30		acg	gac	ggc	gct	gcg	aac	acg	gtc	tgg	acc	aat	gtg	atg	cgc	aac	gac	1872		
		Thr	Asp	Gly	Ala	Ala	Asn	Thr	Val	Trp	Thr	Asn	Val	Met	Arg	Asn	Asp			
				610				615					620							
		ggc	atc	gac	gtc	atc	cat	ctc	atc	aac	ttg	aag	ggc	aac	aac	ggc	agc	1920		
		Gly	Ile	Asp	Val	Ile	His	Leu	Ile	Asn	Leu	Lys	Gly	Asn	Asn	Gly	Ser			
				625			630					635					640			
		tgg	cgc	gac	ccg	gcc	gcg	aca	cca	tcc	acc	ctc	acc	aac	ctg	ccc	gtc	1968		
		Trp	Arg	Asp	Pro	Ala	Ala	Thr	Pro	Ser	Thr	Leu	Thr	Asn	Leu	Pro	Val			
				645							650					655				
40		aag	tac	tac	ctc	ggc	gat	agc	cca	gtg	cct	gca	tcc	gtg	cac	gtt	gcg	2016		
		Lys	Tyr	Tyr	Leu	Gly	Asp	Ser	Pro	Val	Pro	Ala	Ser	Val	His	Val	Ala			
				660					665						670					
		agc	ccc	gac	cga	gac	ggc	ggc	cgt	tcc	acc	gca	ctg	ccc	ttc	acc	acg	2064		
		Ser	Pro	Asp	Arg	Asp	Gly	Gly	Arg	Ser	Thr	Ala	Leu	Pro	Phe	Thr	Thr			
				675				680						685						
45		gga	tcg	gac	gcg	aac	ggc	acg	tac	ctg	agc	ttc	acc	gtg	ccc	gag	ctg	2112		
		Gly	Ser	Asp	Ala	Asn	Gly	Thr	Tyr	Leu	Ser	Phe	Thr	Val	Pro	Glu	Leu			
				690				695					700							
		aag	agc	tgg	gac	ttc	gtc	tac	ctg	ggc	gat	tcc	acc	gcc	ggc	aac	ggc	2160		
		Lys	Ser	Trp	Asp	Phe	Val	Tyr	Leu	Gly	Asp	Ser	Thr	Ala	Gly	Asn	Gly			
				705			710					715					720			
50		aac	gtc	gtc	acc	aag	gcg	agc	aag	tgc	ctc	gac	gtc	gcc	ggc	gga	agt	2208		
		Asn	Val	Val	Thr	Lys	Ala	Ser	Lys	Cys	Leu	Asp	Val	Ala	Gly	Gly	Ser			
				725							730					735				
		gcg	gcg	aac	gga	acc	gct	gtc	cag	atc	tgg	gac	tgc	gcc	agc	gtg	ccc	2256		
		Ala	Ala	Asn	Gly	Thr	Ala	Val	Gln	Ile	Trp	Asp	Cys	Ala	Ser	Val	Pro			
				740						745					750					
55		gcc	cag	gac	tgg	gac	tac	gcg	ggc	gag	aag	ctc	tcc	gcg	ctg	ggc	aag	2304		
		Ala	Gln	Asp	Trp	Asp	Tyr	Ala	Gly	Glu	Lys	Leu	Ser	Ala	Leu	Gly	Lys			

[illegible]

Claims

- 35 1. A cycloisomaltotetraose, having a structure in which 4 D-glucose molecules are bound in cyclic form *via* α -1,6 glucosidic linkages.
2. A crystal of the cycloisomaltotetraose of claim 1.
- 40 3. The crystal of claim 2, which is in the form of a pentahydrate crystal.
4. The crystal of claim 3, exhibiting characteristic diffraction peaks at the diffraction angles (2θ) of 9.0° , 9.8° , 11.8° , 13.1° and 19.1° in its powder X-ray diffraction pattern.
- 45 5. A saccharide composition comprising the cycloisomaltotetraose, which comprises the cycloisomaltotetraose of claim 1 and/or the crystal of claim 2, together with other saccharide(s).
6. The saccharide composition comprising the cycloisomaltotetraose of claim 5, which is in the form of syrup, powder, crystal-containing powder, or solid.
- 50 7. A glucosyl derivative of the cycloisomaltotetraose, having a structure in which one or two D-glucose molecules are bound to the cycloisomaltotetraose.
8. A cycloisomaltotetraose-forming enzyme which has an activity of acting on α -1,6 glucan with a glucose polymerization degree of 4 or higher, dextran, or partial hydrolysate of dextran and forming the cycloisomaltotetraose of claim 1.
- 55 9. The cycloisomaltotetraose-forming enzyme of claim 8, which has the following physicochemical properties:

- (1) Molecular weight
86,000 ± 10,000 daltons on SDS-polyacrylamide gel electrophoresis;
- (2) Optimum temperature
35 to 40°C when reacted at pH 6.0 for 30 min;
- (3) Optimum pH
pH 5.5 to 6.0 when reacted at 40°C for 30 min;
- (4) Thermal stability
Stable up to the temperature of 40°C when incubated at pH 6.0 for 30 min; and
- (5) pH Stability
Stable in a range of pH 5.0 to 10.0 when incubated at 4°C for 16 hours.

10. The cycloisomaltotetraose-forming enzyme of claim 8 or 9, having an amino acid sequence of SEQ ID NO: 5 or SEQ ID NO: 6 as the N-terminal amino acid sequence.

11. The cycloisomaltotetraose-forming enzyme of any one of claims 8 to 10, which has an amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 8, or an amino acid sequence where one or more amino acid residues in SEQ ID NO: 7 or SEQ ID NO: 8 are deleted, replaced, or added without altering the enzyme activity.

12. The cycloisomaltotetraose-forming enzyme of claim 11, which has an amino acid sequence with the homology of 70% or higher against the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 8.

13. The cycloisomaltotetraose-forming enzyme of any one of claims 8 to 12, which is derived from a microorganism.

14. The cycloisomaltotetraose-forming enzyme of claim 13, wherein said microorganism belongs to the genus *Agreia* or the genus *Microbacterium*.

15. The cycloisomaltotetraose-forming enzyme of claim 14, wherein said microorganism belonging to the genus *Agreia* is *Agreia* sp. D1110 (NITE Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Accession No. NITE BP-02815) or a mutant thereof, and said microorganism belonging to the genus *Microbacterium* is *Microbacterium trichothecenolyticum* D2006 (NITE Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Accession No. NITE BP-02816) or a mutant thereof.

16. A microorganism capable of producing the cycloisomaltotetraose-forming enzyme of any one of claims 8 to 15, which is *Agreia* sp. D1110 (NITE Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Accession No. NITE BP-02815) or a mutant thereof or *Microbacterium trichothecenolyticum* D2006 (NITE Patent Microorganisms Depositary, National Institute of Technology and Evaluation, Accession No. NITE BP-02816) or a mutant thereof.

17. A DNA, which encodes the cycloisomaltotetraose-forming enzyme of claim 11.

18. The DNA of claim 17, which comprises a nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10, a nucleotide sequence where one or more nucleotides in SEQ ID NO: 9 or SEQ ID NO: 10 are deleted, replaced, or added without altering the encoded enzyme activity, or complementary nucleotide sequences thereof.

19. The DNA of claim 18, which is obtainable by replacing one or more nucleotides of SEQ ID NO: 9 or SEQ ID NO: 10 without altering the amino acid sequence encoded thereby based on the degeneracy of genetic code.

20. The DNA of any one of claims 17 to 19, which is derived from a microorganism of the genus *Agreia* or the genus *Microbacterium*.

21. A replicable recombinant DNA, which comprises the DNA of any one of claims 17 to 20 and an autonomously replicable vector.

22. A transformant, which is obtainable by introducing the recombinant DNA of claim 21 into an appropriate host.

23. A process for producing a cycloisomaltotetraose-forming enzyme, comprising the steps of:

culturing a microorganism capable of producing the cycloisomaltotetraose-forming enzyme of any one of claims

8 to 15 in a nutrient culture medium; and
collecting the cycloisomaltotetraose-forming enzyme from the resulting culture.

24. A process for producing a recombinant cycloisomaltotetraose-forming enzyme, comprising the steps of:

culturing the transformant of claim 22; and
collecting a recombinant cycloisomaltotetraose-forming enzyme from the resulting culture.

25. A process for producing the cycloisomaltotetraose of claim 1, the crystal of the cycloisomaltotetraose of claim 2, or the saccharide composition comprising cycloisomaltotetraose of claim 5 or 6, comprising a step of allowing the cycloisomaltotetraose-forming enzyme of any one of claims 8 to 15 to act on a solution containing α -1,6 glucan with a glucose polymerization degree of 4 or higher, dextran, or a partial hydrolysate of dextran.

26. A process for producing the cycloisomaltotetraose of claim 1, the crystal of the cycloisomaltotetraose of claim 2, or the saccharide composition comprising cycloisomaltotetraose of claim 5 or 6, comprising a step of allowing an enzyme, which has an activity of acting on starch or a partial starch hydrolysate and forming α -1,6 glucan with a glucose polymerization degree of 4 or higher, dextran, or a saccharide having α -1,6 glucan structure as a part; and the cycloisomaltotetraose-forming enzyme of any one of claims 8 to 15 to act in combination on a solution containing starch or a partial starch hydrolysate.

27. A composition comprising the cycloisomaltotetraose of claim 1, the crystal of the cycloisomaltotetraose of claim 2, the saccharide composition comprising cycloisomaltotetraose of claim 5 or 6, or the glucosyl derivative of cycloisomaltotetraose of claim 7, together with other ingredient(s).

28. The composition of claim 27, which is in the form of foods and beverages, luxury grocery items, feeds, baits, cosmetics, pharmaceuticals, or industrial supplies.

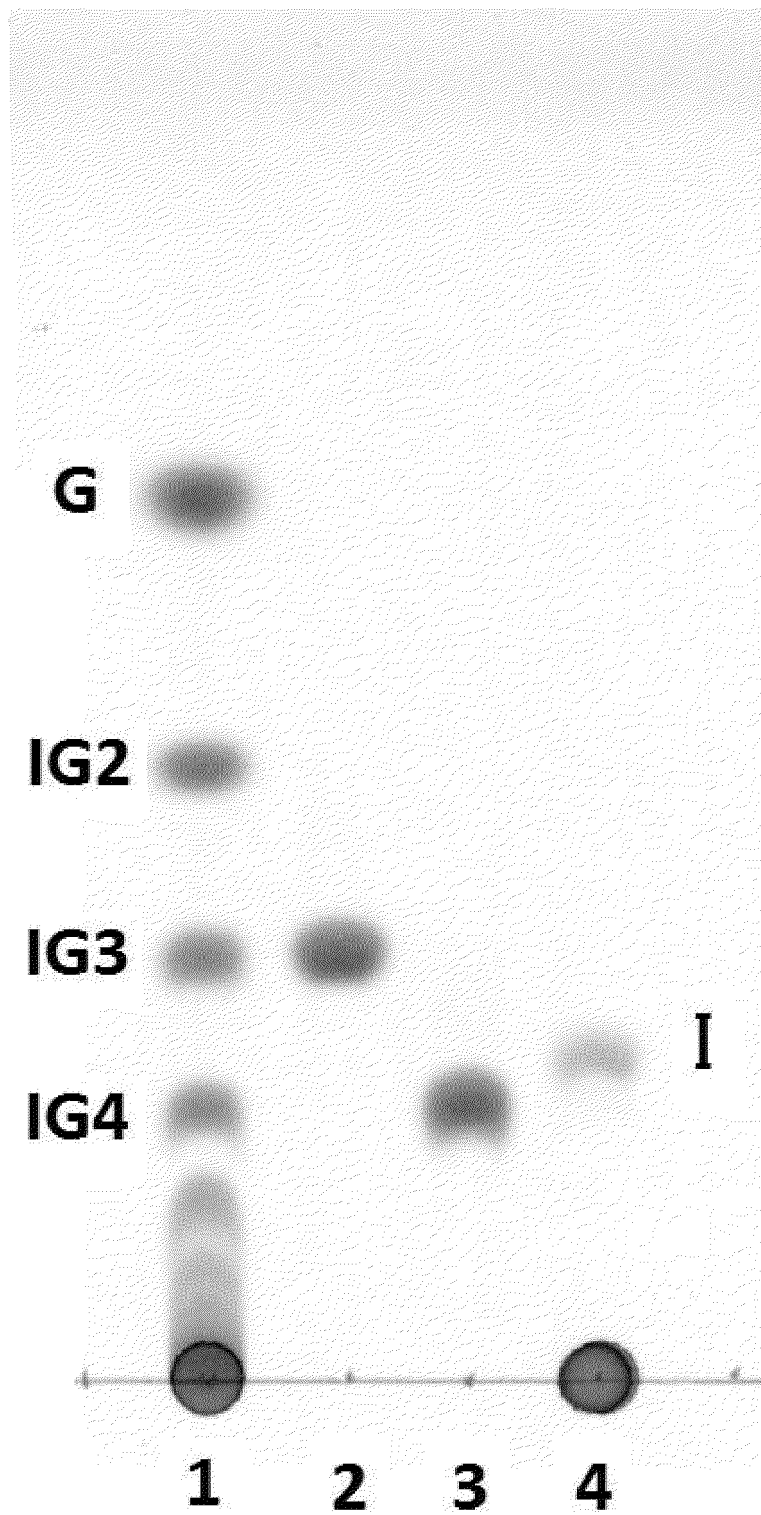


FIG.1

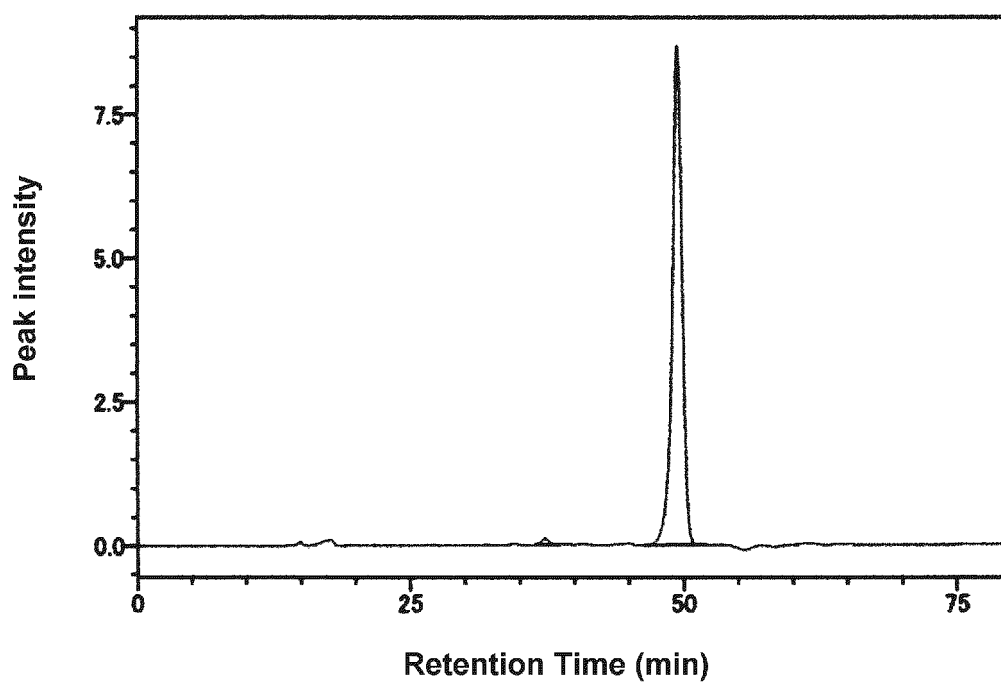


FIG.2

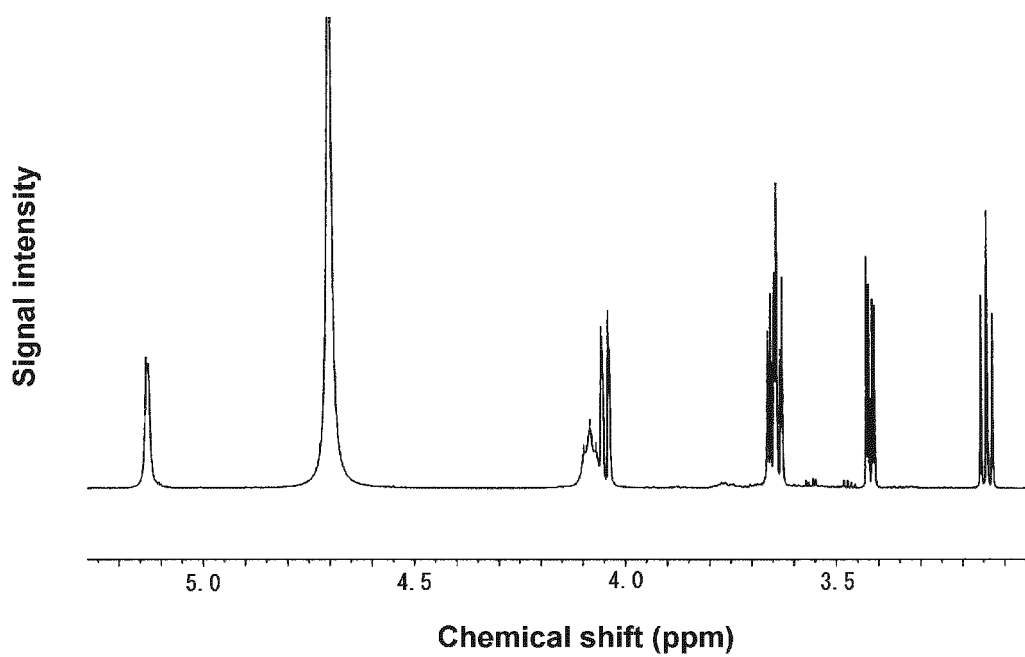


FIG.3

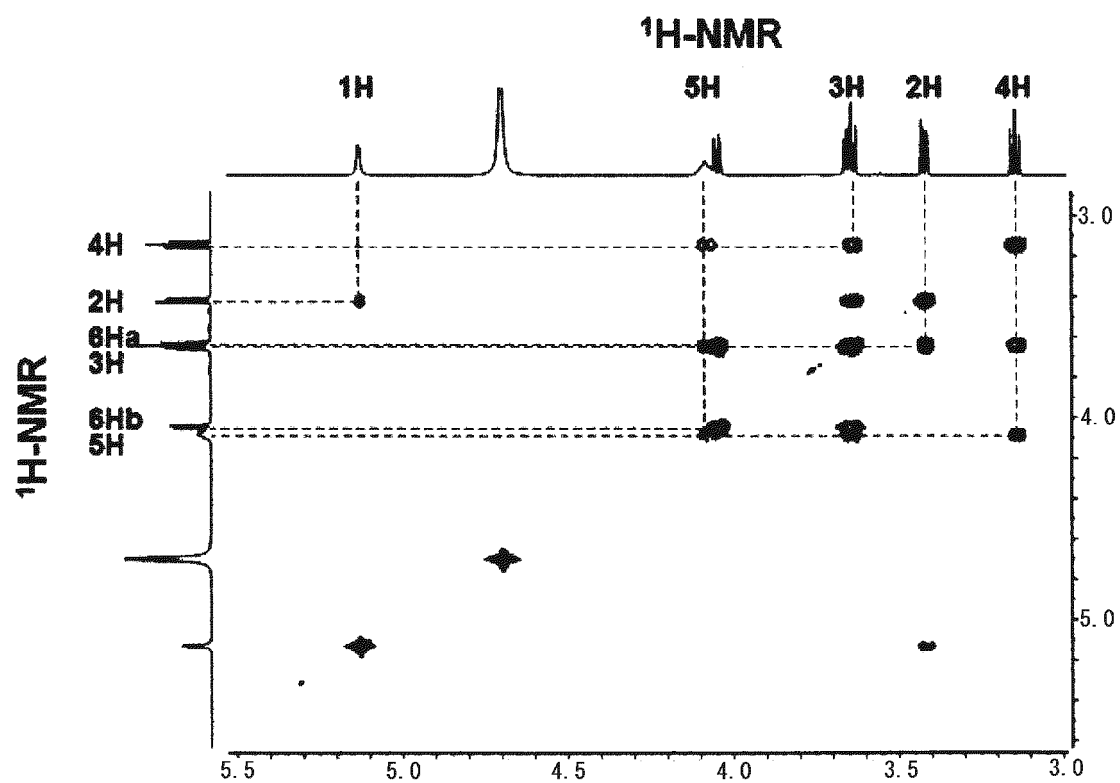


FIG.4

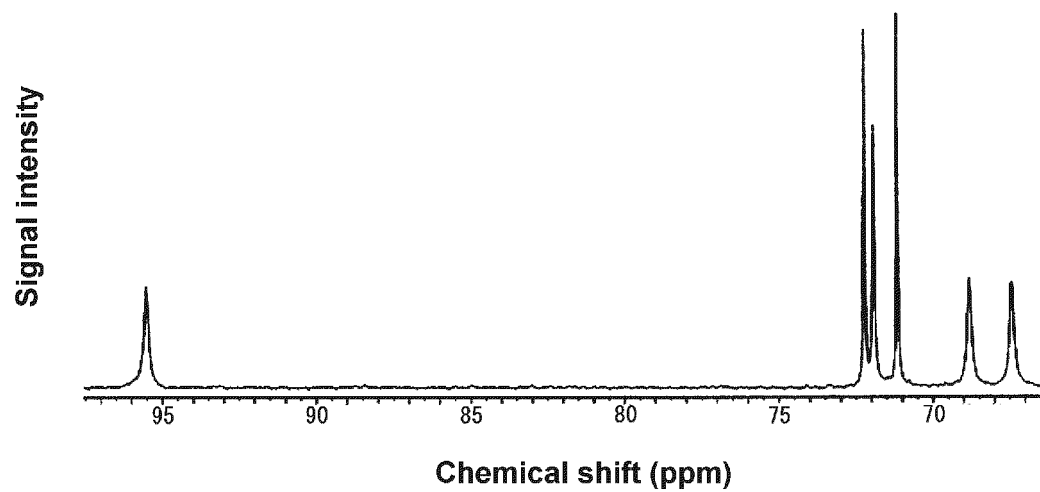


FIG.5

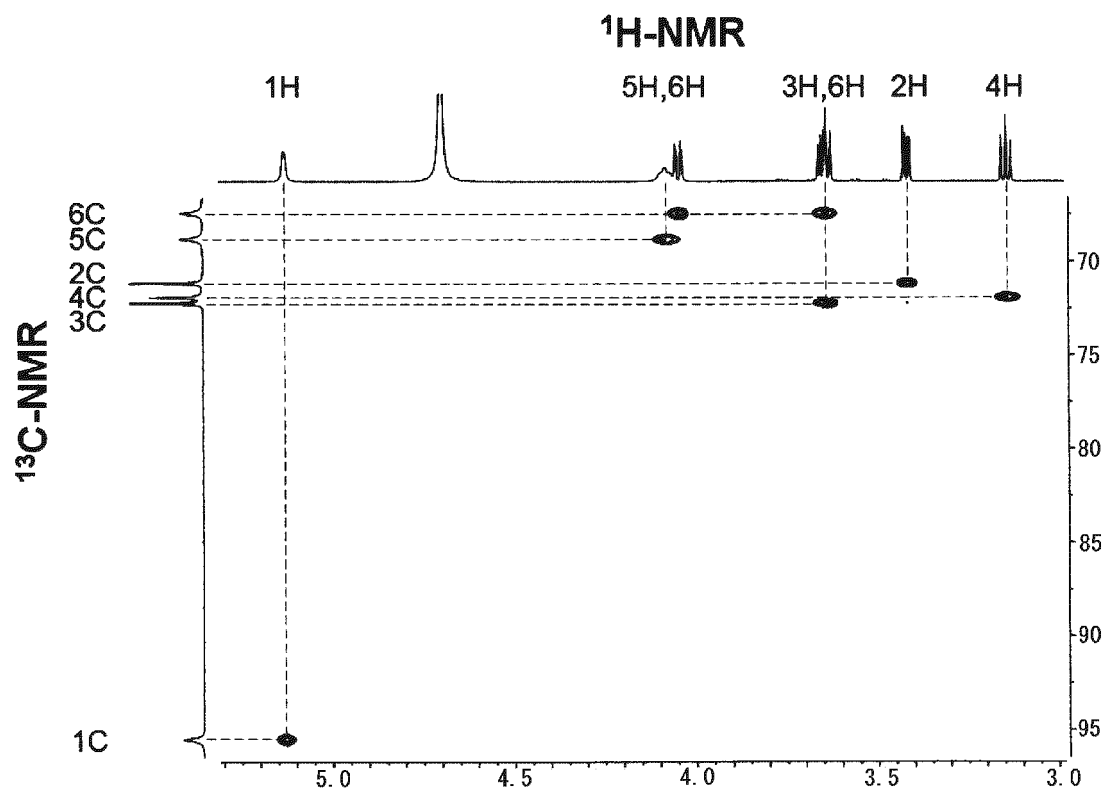


FIG.6

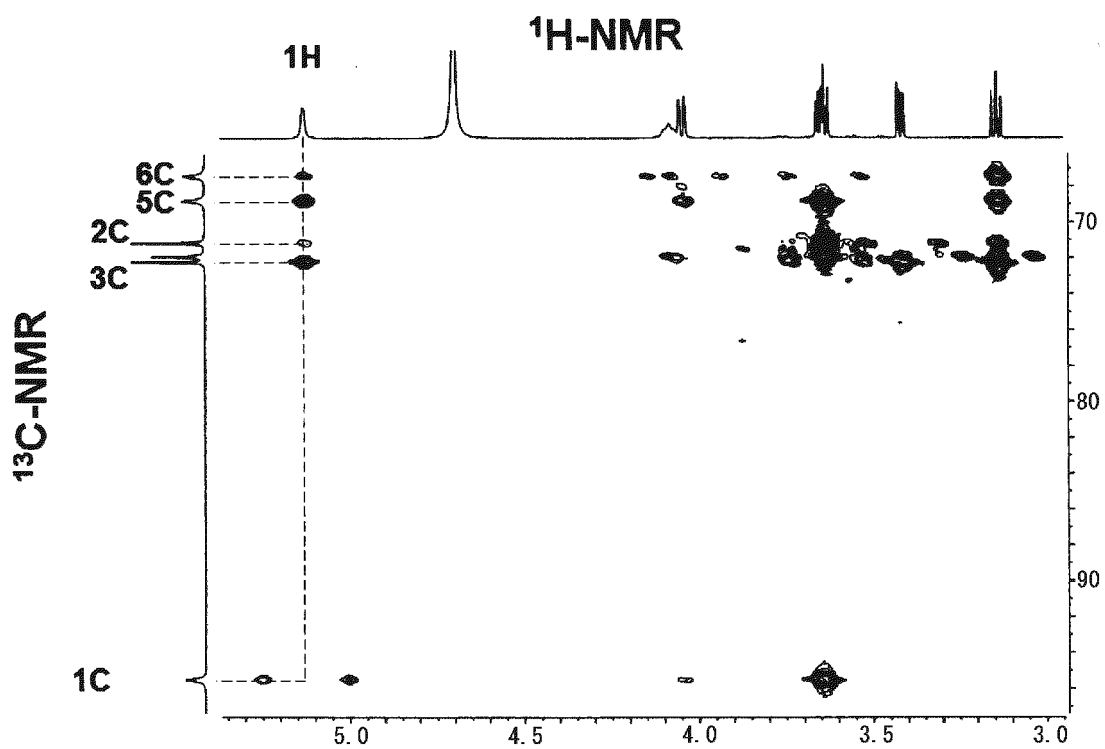


FIG.7

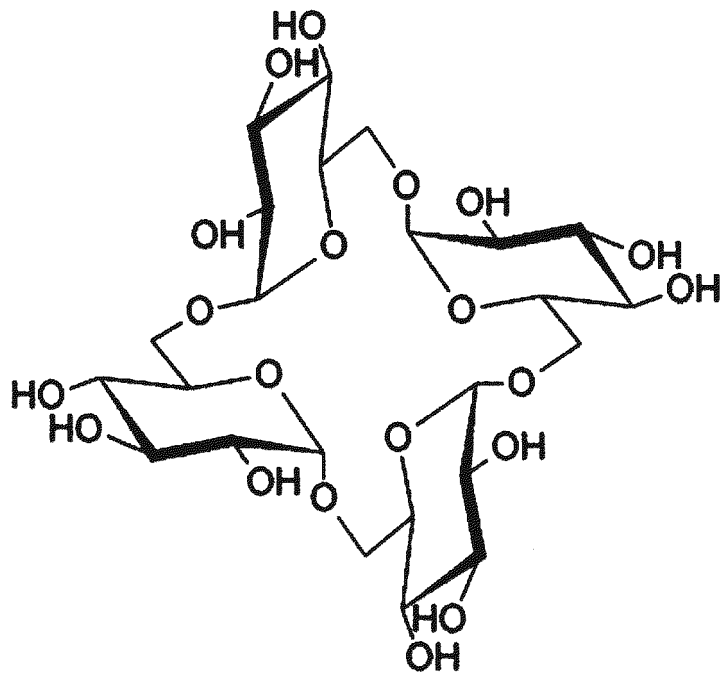


FIG.8

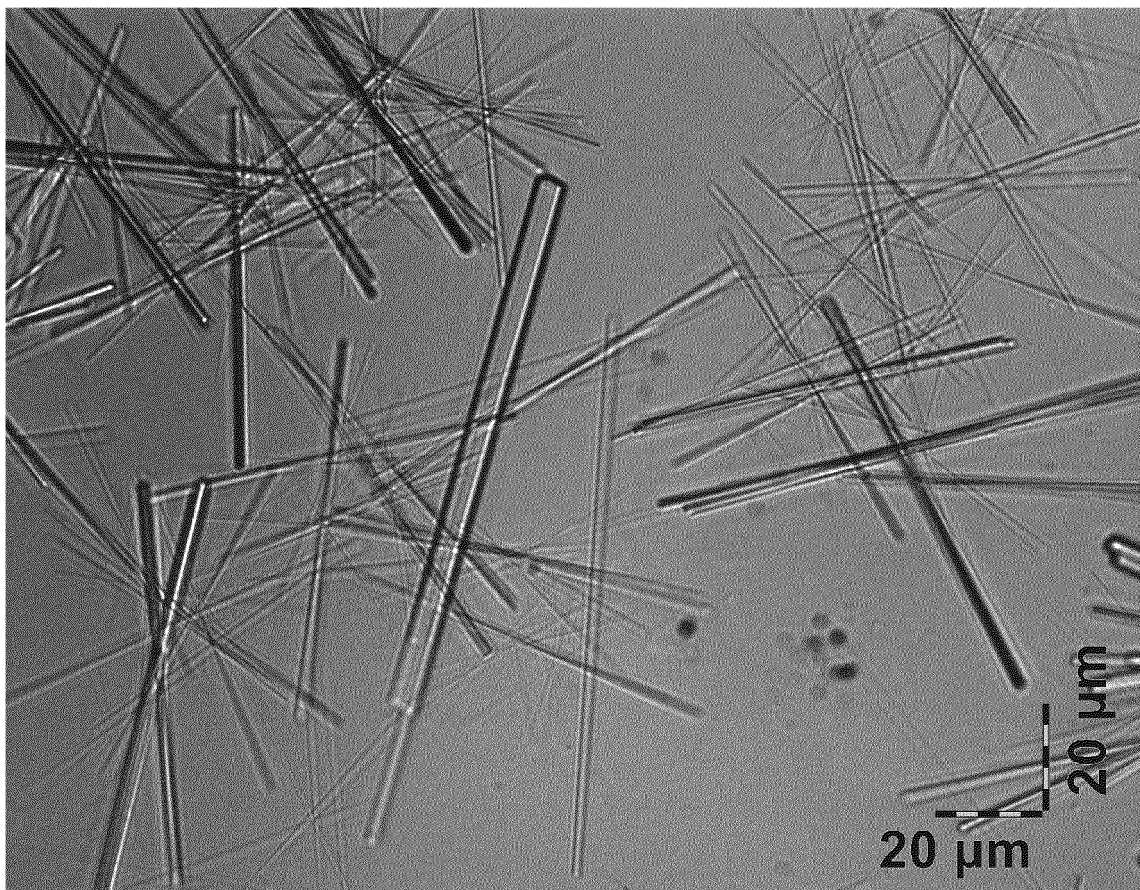


FIG.9

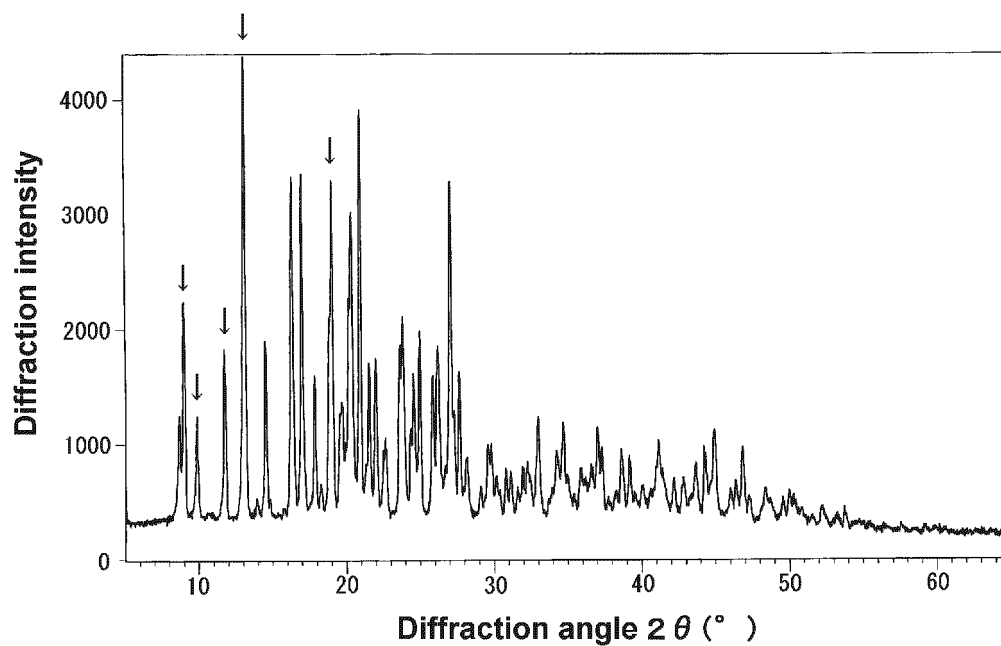


FIG.10

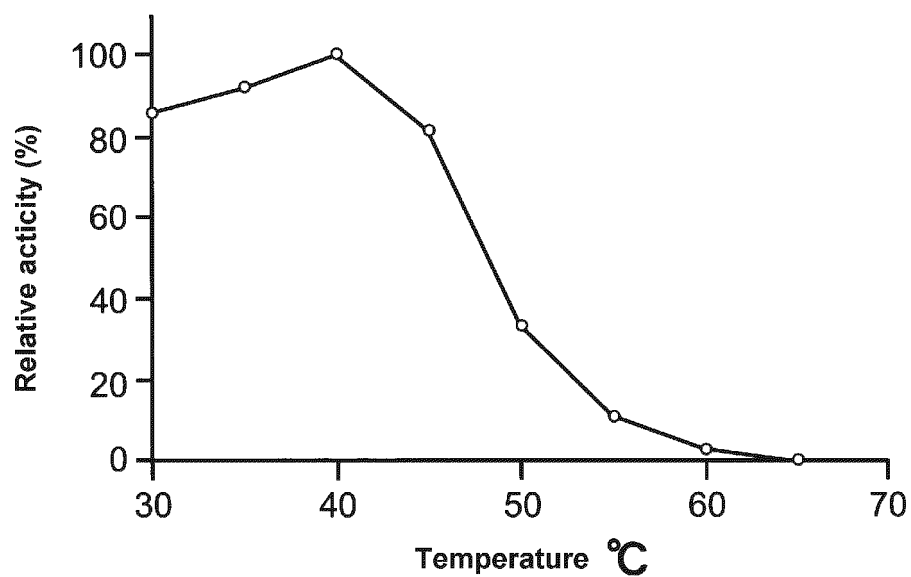


FIG.11

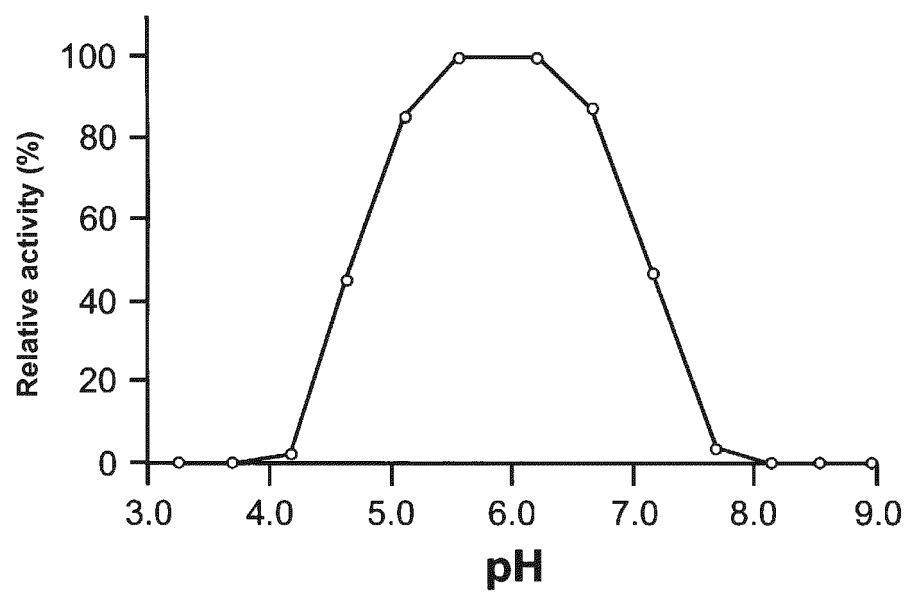


FIG.12

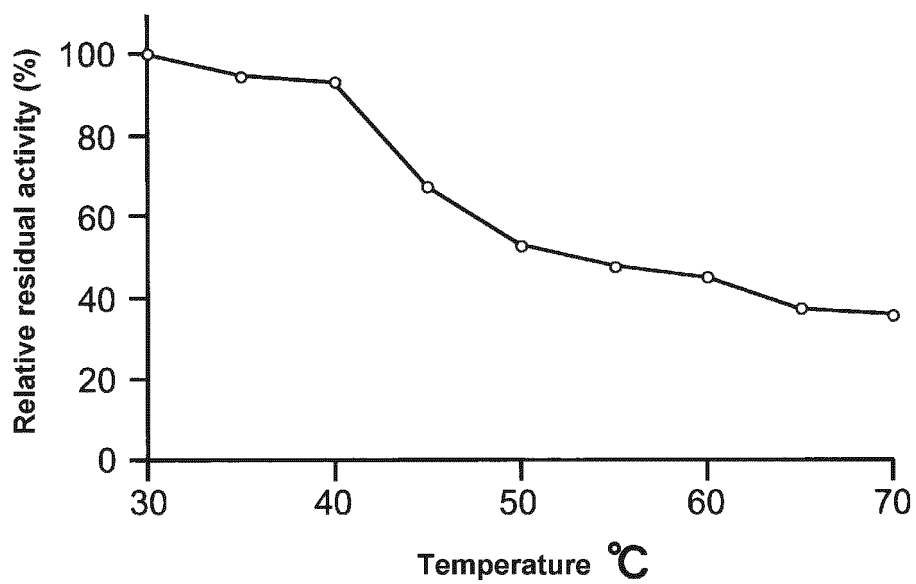


FIG.13

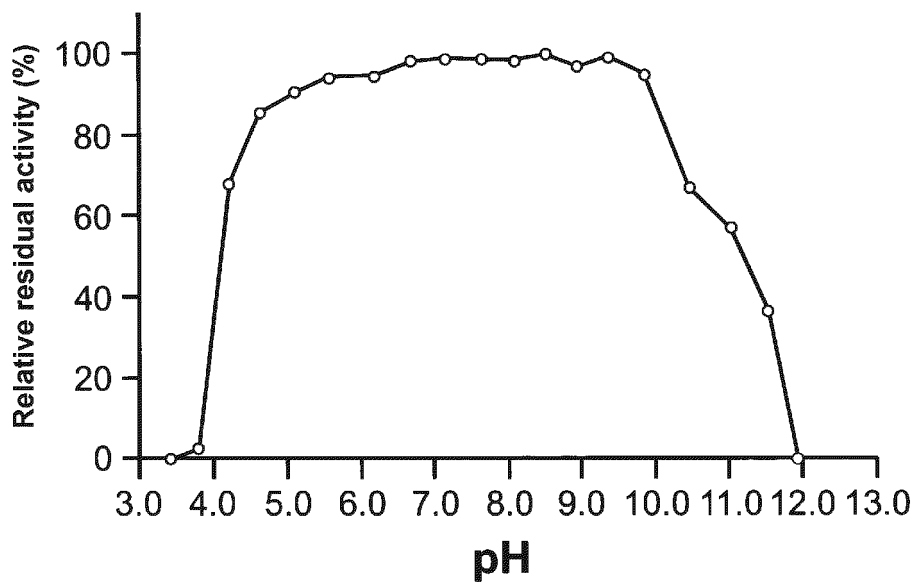


FIG.14

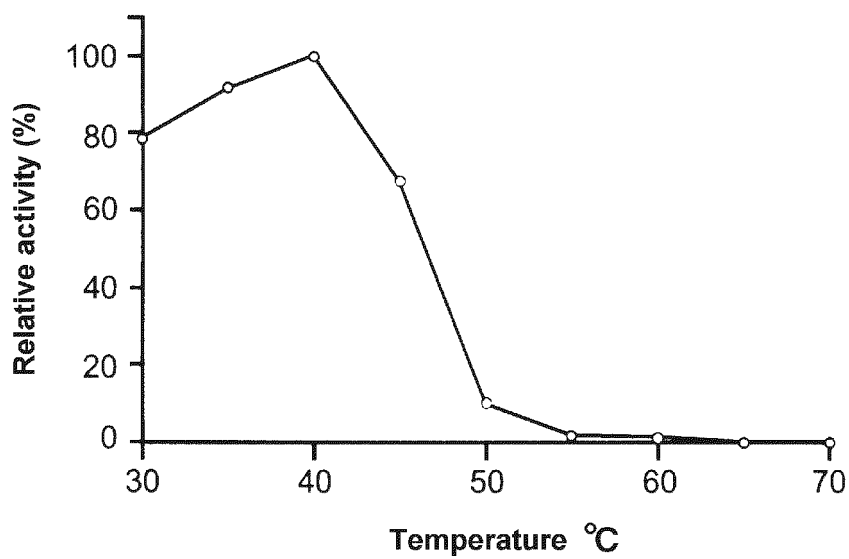


FIG.15

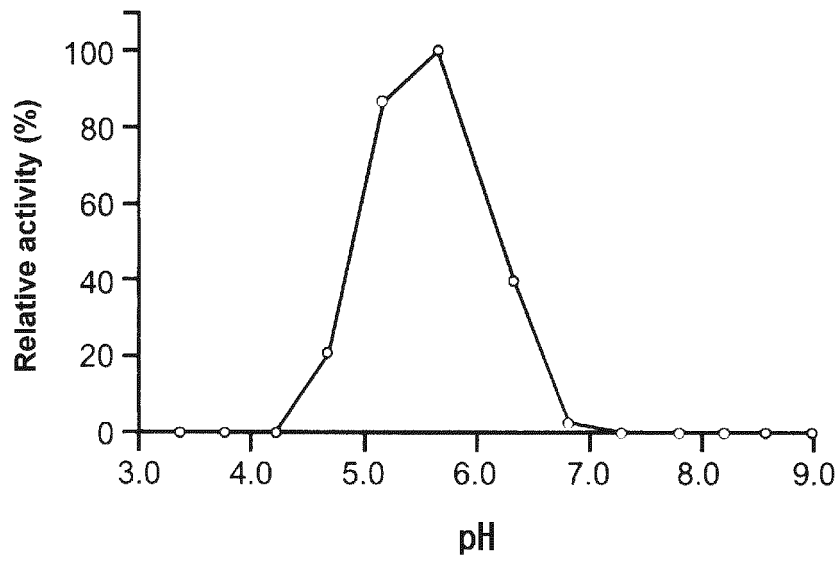


FIG.16

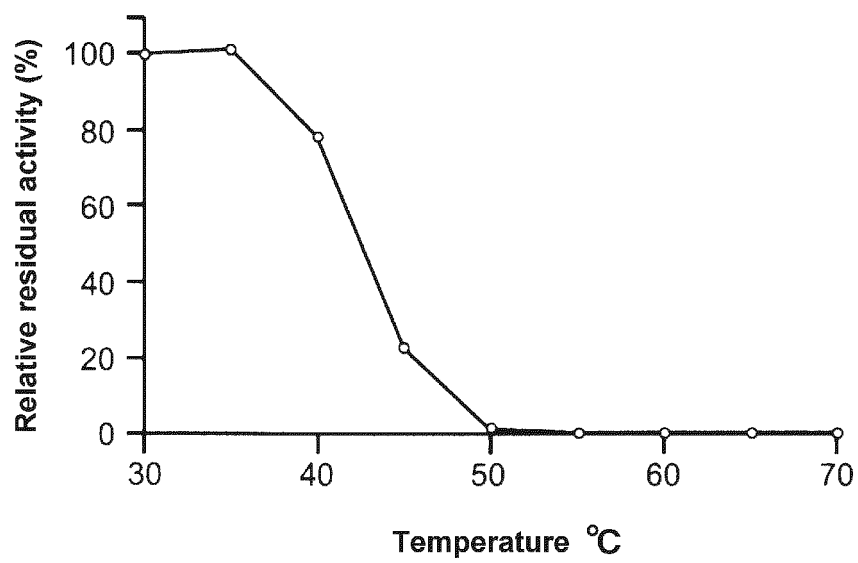


FIG.17

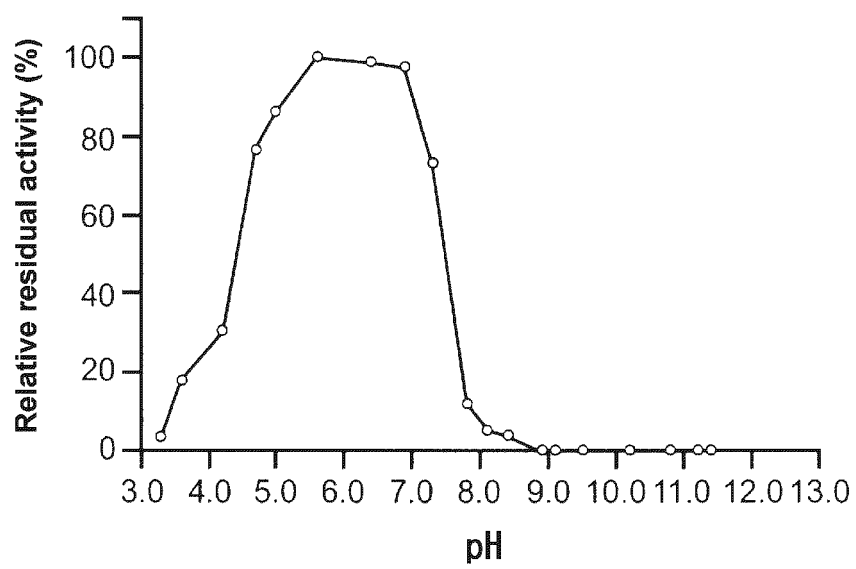


FIG.18

ORF9038	1	-MKSRRFRRIIGACGTAALIAAGVVLPAVPASAAITVGDWTDKARYDPGQVTVTAEVDCS	59
ORF5328	1	MRSSPFRRRIITAVTTIAGLAVAGIIVPAAPAAADLGDAWTDKARYAPGQVTVTAEVSGT	60
ORF9038	60	GPVEFSLVHLGETVIRGTVQATGSGGIVITWTVTPPTTDFGTGLVHADAGGSTAQTAVDVSS	119
ORF5328	61	GSVQFSLVHLGDVVDTGTVTIATGSGGQVNWTVTPPANDFTGYMVHVDAGSSTIQTAVDVSS	120
ORF9038	120	ITWTRFPRMGYLLDEYFADSTQADRDALVITDLANKYHLNALQFYDWMWRHEAPLERDGNGLD	179
ORF5328	121	NWTRFPRTIGFLDTYPIQLDSTEQAATVDELSRNYHLNSLQFYDWMWRHEKEPVQRESNGDL	180
ORF9038	180	VGTTWTAWNGDVIAPATVSGLVDAHVNVAALPYSMSYAALEGFEAHGVDADWRLKYRSD	239
ORF5328	181	VDTWTAWNGDVIAPDTVAGLIDATHDVNAAALPYSMSYAALEGFOAHGVDADWRLKYRSS	240
ORF9038	240	SSDWKFQMLEGRPDINLWIMNPNPDWRDHISAEYDQDIDTFGFDGTHLDQLGNWGS GAS	299
ORF5328	241	GDDWKQMLEPNEPDITLWIMNPNPDWVSHITEQYADQIDALGFDGTHLDQLGNWGEAV-	299
ORF9038	300	DGGMNDVAGNFPVDIPVGFRLVATKKAATGKPVGFNAVDGFAGSTLASSSDYLYTELWE	359
ORF5328	300	DGGMDDVAGDPVDIPQGFANLVATKKAATGKTTFGFNAVDGFGSDWLAGESEDYLYTELWE	359
ORF9038	360	NHETYSEVQSYLAGQRAESGGKAAVIAAYLNYSNTGDRYEAEQDILIG-VQVNSNHPGY	418
ORF5328	360	NHETYAQVQDYLAQORAASGGKGAVLAAYLNYSNTGDOYEAEAGVLAGGVQVDSQHTGY	419
ORF9038	419	TGTGFVDQFGDVGDSVTLSVIVPESRRYGVPAWANDTATATRTVRVDGVPAGKLMTP	478
ORF5328	420	TGTGFILDSYGASGDVITVIVAVPESRRYGVPRWINGTGATATRTVIVDGTNVGRIKLPS	479
ORF9038	479	TGSDWSWNIEAGFGTYLSAGSHITIEVSLDAGDTGFANLDSITLGTFNTPSVQLANAALAA	538
ORF5328	480	TADWDIWNIEGGTAAYLIAGTHTITLAVGSGDTGYVNLDSITLGTFDTPSVQLANAFAA	539
ORF9038	539	SGASHIEMGQGDQMLVAPYFLDDSKOMSTELQAMMESYYDVITGYENILYGPTRLQLPNA	598
ORF5328	540	NGASHIEMGQGDQMLVAPYFLDETKOMNHELQMLEKYYDVITGYENILFYGPTRLQLSNA	599
ORF9038	599	VSTAGHTTSTDGTGDTLWATPVRNDGMVDVHLINLEGNDCRWREPAAQAPTLSNLPVKYY	658
ORF5328	600	VITLSCQPTSTDGAANIVMTNVMRNDGIDVHLINLKGNNGSWRDPAATPSTLINLPVKYY	659
ORF9038	659	LADRPVPADVRIASPD-NGGRSSTLAFTSGSDAGGNYISFTVPELKNWSFVYLGDSIRGN	717
ORF5328	660	LGDSPPVPASVHVASPD RDGGRSTALPFTTIGSDANGIYLSFTVPELKSMDFVYLGDSIAGN	719
ORF9038	718	GNVVTQASTCLDVAGGNSADGTAVQVMNCASVPAMQMTFQNNILSALGKCLDLVAGGTAD	777
ORF5328	720	GNVVTIKASKCLDVAGGSAANGTAVQIWD CASVPAQDWDYAGEKLSALGKCLDLVYQGGTAN	779
ORF9038	778	GTLAHLWICSDVPSQIMERTGQGGYLNPAASGKCLDLVGGSTANGTRAHLWSCHAGASQKW	837
ORF5328	780	GTLAHLWDGANVPSQGWRTAQSQYINPAASGRCLDLVGGGAANGTRIHLWDCHAGPSQKW	839
ORF9038	838	TLPQ	841
ORF5328	840	TLPG	843

FIG.19

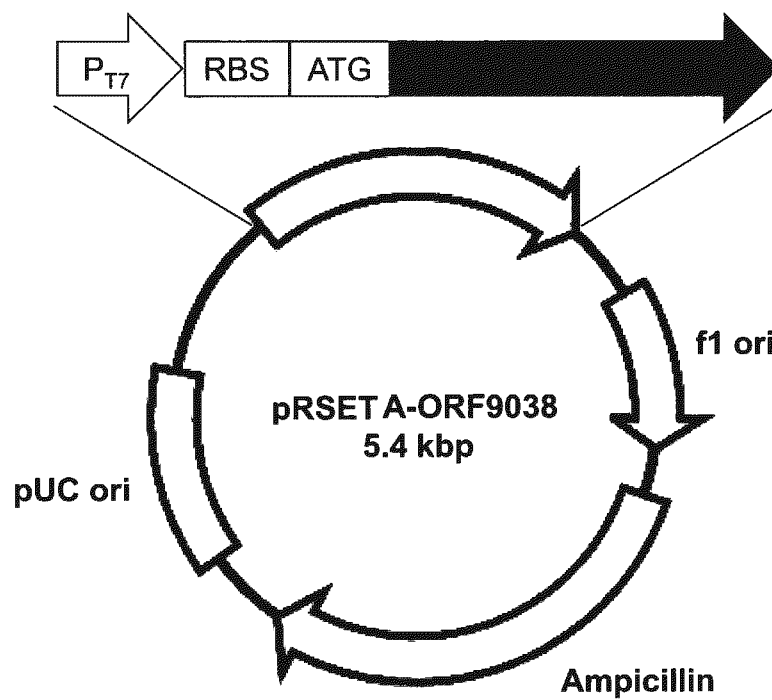


FIG.20

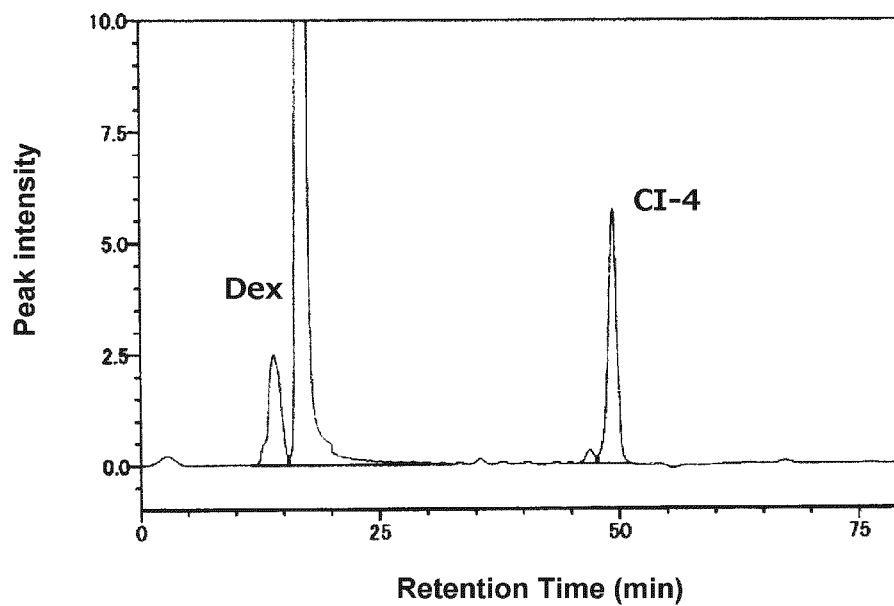


FIG.21

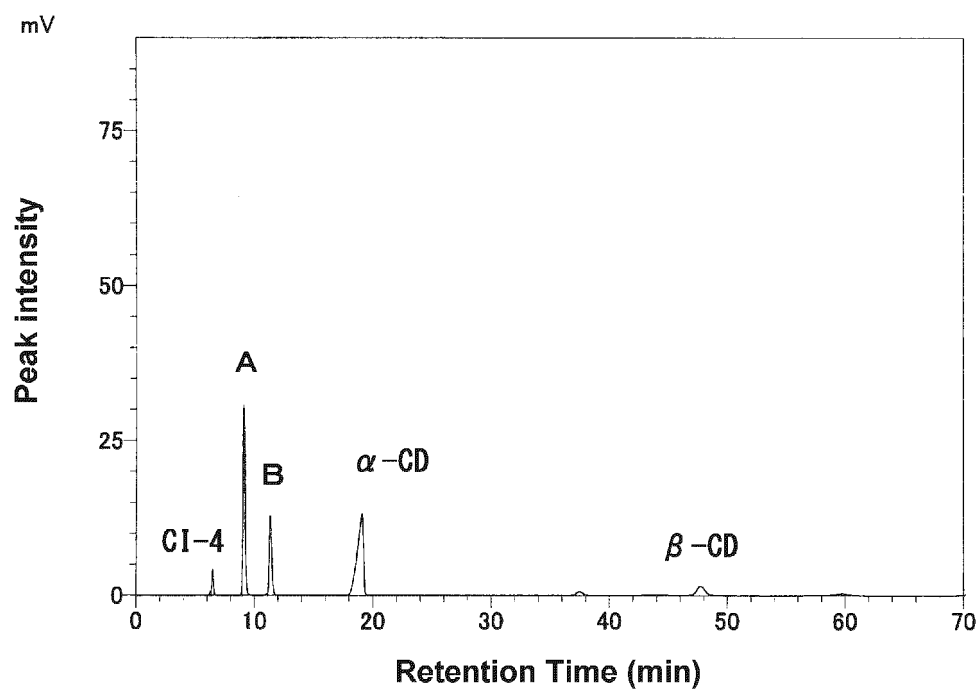


FIG.22

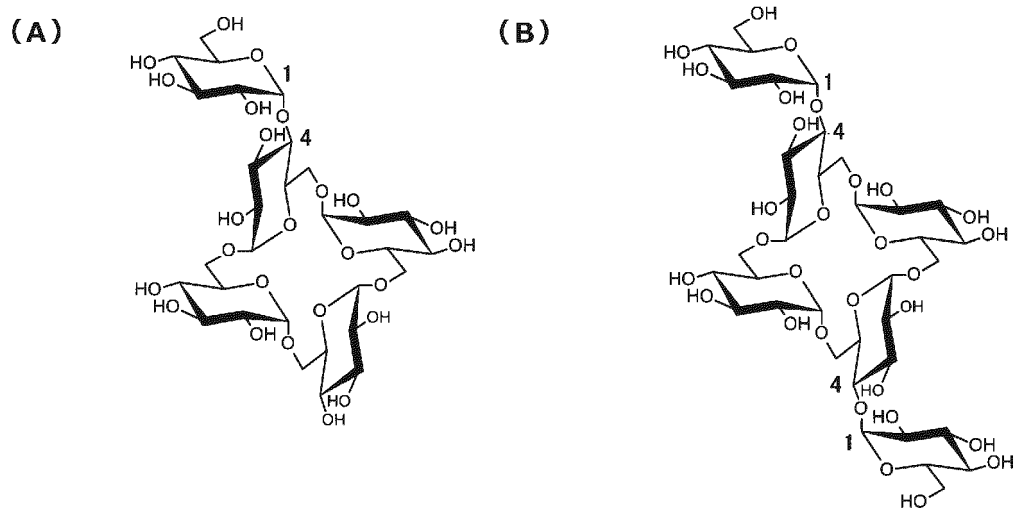


FIG.23

5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2019/048218

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. A61K31/724(2006.01)i, C12P19/18(2006.01)i, C12R1/00(2006.01)n, C12N5/10(2006.01)i, A23K20/163(2016.01)i, A23L33/125(2016.01)i, A61K8/73(2006.01)i, A61K47/40(2006.01)i, G12N1/15(2006.01)i, C12N1/19(2006.01)i, C12N1/20(2006.01)i, C12N1/21(2006.01)i, G12N15/52(2006.01)i, C08B37/16(2006.01)i, C12N9/00(2006.01)i
 FI: C12P19/18, C08B37/16 ZNA, C12N1/15, C12N1/19, C12N1/21, C12N9/00, C12N1/20 A, C12N15/52 Z, G12N5/10, A23L33/125, A61K8/73, A61K47/40, A61K31/724, A23K20/163, C12R1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl. A61K31/724, C12P19/18, C12R1/00, C12N5/10, A23K20/163, A23L33/125, A61K8/73, A61K47/40, C12N1/15, G12N1/19, G12N1/20, C12N1/21, G12N15/52, G08B37/16, G12N9/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
 Published unexamined utility model applications of Japan 1971-2020
 Registered utility model specifications of Japan 1996-2020
 Published registered utility model applications of Japan 1994-2020

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST7580 (JDreamIII); REGISTRY/CAPLUS/MEDLINE/EMBASE/BIOSIS/WPIDS (STN); GenBank/EMBL/, DDBJ/GeneSeq; UniProt/GeneSeq

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Cote G. L. and Biely P., Enzymically produced cyclic alpha-1, 3-linked and alpha-1, 6-linked oligosaccharides of D-glucose, European journal of biochemistry, 01 December 1994, vol. 226, no. 2, pp. 641-648, pp. 641-648 abstract, schemes 1, 2, fig. 5, 6	1-28
A	渡邊光, オリゴ糖研究の最前線 その1 環状オリゴ糖の応用研究環状四糖の機能, 応用糖質科学, 2011, vol. 1, no. 4, pp. 307-312, sections 1, 2, fig. 1-3, section 6, non-official translation (WATANABE, Hikaru. The Front Lines of Oligosaccharide Research: Part 1, Cyclic Oligosaccharide Applied Research Cyclic Tetrasaccharide Functions. Bulletin of Applied Glycoscience.)	1-28 1-28

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
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